ol. 7
961

Biochemical Pharmacology

VOLUME 7 1961

EDITORIAL BOARD

Chairman
ALEXANDER HADDOW

Vice-Chairmen
SIDNEY FARBER Z. M. BACQ

Regional Editors
A. D. WELCH (New Haven) RUDOLPH A. PETERS (Cambridge)
M. WELSCH (Liège) P. ALEXANDER (London–*Executive Editor*)

Supported by an International
Honorary Editorial Advisory Board

Copyright © 1961
Pergamon Press Ltd

PERGAMON PRESS

Oxford · London · New York · Paris

AIMS

THIS international journal will be devoted to research into the development of biologically active substances and their mode of action at the biochemical and sub-cellular level.

Full length papers will be published approximately four months, and short communications in six to eight weeks, after final acceptance.

In addition to original research papers, the journal will offer immediate publication for Short Communications. Letters to the Editors, offering comment on previous articles, will also be accepted, when the contribution is considered warranted by the Editors.

The journal will include reports on all types of cells (plant, micro-organism and animal), but particular emphasis will be given to cancer chemotherapy and related studies. It is hoped that research will be stimulated as a result of collecting together in one publication investigations from different fields having as a common link the same fundamental problems.

The new journal will provide a forum for the publication of all phases of pure and applied cellular pharmacology including organochemical, physicochemical, biochemical and cytological studies. By including all aspects of research involved in the development of biologically active materials (chemotherapeutic agents as well as insecticides, fungicides, plant growth regulators, etc.), it is hoped that a better appreciation of the problems involved will be attained. In the past the different research approaches which make up these investigations have almost invariably been reported in a number of widespread publications and the interplay of different types of investigations towards solving the main problem has thereby been available only with difficulty to workers in neighbouring fields. *Biochemical Pharmacology* is intended to remedy this defect and to provide a means of viewing the entire field in its proper perspective.

NOTES FOR CONTRIBUTORS

Submission of papers

Original contributions (which may be in English, French or German) should be sent direct to the regional editors. Papers from the U.S.A. and Canada should be addressed to Prof. A. D. WELCH, those from Europe to Prof. M. WELSCH, and those from the United Kingdom to Dr. P. ALEXANDER.

Note: The contents of papers shall be the sole responsibility of the authors and publication shall not imply the concurrence of editors or publishers.

Proofs

Proofs will be sent to authors for correction when their contribution is first set, but there is rarely time for page proofs also to be sent for checking. This will be done however, where the amount of alteration makes it advisable.

Reprints

Fifty free reprints will be provided and additional reprints may be obtained at reasonable cost; a reprint order form will be supplied with galley proofs.

Form of contribution

Scripts should, if possible, be submitted in duplicate. They must be typewritten with lines double spaced. Contributions should be prepared in the form generally accepted by the learned societies and an Abstract, an Introduction, Experimental Section, Results and Discussion (or Summary) will be the normal practice, although these may be linked together, i.e. Results and Discussion, or Experimental and Results.

Symposia and Conference Reports

From time to time critical summaries will be published of the proceedings of conferences and symposia held on a specific topic pertinent to those interested in the biochemical aspects of pharmacology. Suggestions for conferences which should be reported are most welcome and should be addressed to the regional editors. It is hoped to publish reports of this type within about three months of the time of the conference.

Abstracts

The essential contents of each paper should be briefly recapitulated in an abstract. This should be in the language of the paper, but for French or German papers an English version should also be provided wherever possible.

Nomenclature

This is to follow internationally agreed rules apart from a few instances which may lead to illogical or meaningless results. Nomenclature following international commission rules will always be accepted, however, and not modified, the Editors' function being advisory in this matter.

Illustrations

Illustrations should be separate from the typescript of the paper and legends should also be typed on a separate sheet. Line drawings which require re-drawing should include all relevant details and clear instructions for the draughtsman. If the figures are already well drawn it may be possible to reproduce them direct from the originals, or from good photoprints if these can be provided. It is not possible to reproduce from prints with weak lines. Illustrations for reproduction should normally be about twice the final size required. A special feature of this publication is careful reproduction of photographs and it is requested that every effort be made to provide good originals. The following standard symbols should be used on line drawings since they are easily available to the printers:

▲ △ ⊡ □ ● ○ ○ ⊕ ⊖ ó ⊗

Tables

Tables should be so constructed as to be intelligible without reference to the text. Every table and column should be provided with an explanatory heading. Units of measure must always be clearly indicated. The same data should not be reproduced in both tables and figures.

References

The system of references is by consecutive superscript numbers in the text, the full reference being cited in a numbered list at the end of the text. References should contain the names of *all* the authors of any one paper together with their initials, the title of the journal (abbreviated if possible in accordance with the *World List of Scientific Periodicals*), volume number, first page number and year, in the following way:

1. H. KEWITZ, I. B. WILSON and D. NACHMANSOHN, *Arch. Biochem. Biophys.* **64**, 456 (1956).
2. S. T. YOFFE and A. N. NESMEYANOV, *Handbook of Magnesium-Organic Compounds* p. 380. Pergamon Press, London (1956).

Footnotes

Footnotes, as distinct from literature references, should be indicated by the following symbols: *, †, ‡, §, commencing anew on each page; they should not be included in the numbered reference system.

Vol. 7
1961

NUMBER 1

P. Scriba, H. Kröger und H. Holzer: Wirkung carcinostatischer Äthylenimin-Verbindungen auf den DPN-Gehalt therapieresistener Tumoren . . . 1
E. D. Wills: Effect of unsaturated fatty acids and their peroxides on enzymes . . . 7
S. Chaube and C. E. Carter: The effect of chlorthiazide on hypoxanthine dehydrogenase, lactic dehydrogenase, and soluble protein of chick embryo kidney . . . 17
A. Jeney, A. Szendrey et E. Vig: Détermination des acides nucléiques de *Saccharomyces italicus* (Castelli) traités par l'oxyde de carbone (CO), le cyanure de potassium (CNK) ou l'azide de sodium (N_3Na) . . . 23
R. U. Lemieux, K. F. Sporek, I. O'Reilly and E. Nelson: The oxidation of N-*p*-benzotrideuteridesulfonyl N′-*n*-butylurea (tolbutamide-D_3) by man . . . 31
D. Jamieson and H. A. S. van den Brenk: Relation of mast cell changes to hypothermia in the rat . . . 35
A. Horita and L. J. Weber: The enzymic dephosphorylation and oxidation of psilocybin and psilocin by mammalian tissue homogenates . . . 47
O. W. Wendel and E. M. Brophy: Experimental bowel shock in rats—II. Liver nucleotide changes . . . 55
Mary L. C. Bernheim: The effect of carbon tetrachloride, ethionine and chloretone on the ascorbic acid content of rat liver . . . 59
Clarke Davison, Robert S. Rozman and Paul K. Smith: Metabolism of bis-β-chloroethyl sulfide (sulfur mustard gas) . . . 65

Short Communications:

Glenn A. Fischer: Increased levels of folic acid reductase as a mechanism of resistance to amethopterin in leukemic cells . . . 75
M. Goldstein and J. F. Contrera: The inhibition of norepinephrine and epinephrine synthesis *in vitro* . . . 77
G. G. Villela, O. R. Affonso and E. Mitidieri: Effect of colchicine on the intestinal xanthine oxidase . . . 78

NUMBER 2

Hans Soep and Paul A. J. Janssen: The influence of Haloperidol and chemically related neuroleptics on free amino acid content of rat brain . . . 81
George L. Ellman, K. Diane Courtney, Valentino Andres, Jr. and Robert M. Featherstone: A new and rapid colorimetric determination of acetylcholinesterase activity . . . 88
Bernard H. Marks and Robert W. Sorgen: Effects of reserpine and colchicine on enterochromaffin cells . . . 96
M. Rabinovitz and J. M. Johnson: Synthesis of 4-thiamethionine and its effect on energy metabolism and amino acid incorporation into protein of Ehrlich ascites tumour cells . . . 100
D. J. Lawford: An abnormal serum component in experimental poisoning by cadmium and other metals . . . 109
Harris Busch, Dolores C. Firszt, Allan Lipsey, Elli Kohen and S. Amer: Inhibition by anti-tumour agents of labeling of nuclear proteins *in vivo* with L-arginine-U-^{14}C . . . 123
M. W. Whitehouse and H. Boström: Studies on the action of some anti-inflammatory agents in inhibiting the biosynthesis of mucopolysaccharide sulphates . . . 135
R. A. Steggle, A. K. Huggins and M. J. H. Smith: Inhibition of rat serum glutamic-pyruvic transaminase *in vitro* by congeners of salicylate . . . 151
David A. Karnofsky and Corinne R. Lacon: Effects of physiological purines on the development of the chick embryo . . . 154
Announcement . . . 159
Papers to be published in future issues . . . 160

NUMBERS 3/4

Donn L. Smith and Edward D. Harris, Jr.: The reduction of triphenyltetrazolium by hypertrophied tissues . . . 161
U. Bardi, Giulia Boretti and A. di Marco: Inhibition of oxidative phosphorylation by aminosidine and other antibiotics, in rat liver mitochondria . . . 165
Artur Ber and Rina Singer-Altbeker: The influence of thiamine and related compounds on the iris of the frog . . . 169
R. G. Wiegand and J. E. Perry: Effect of L-DOPA and N-methyl-N-benzyl-2-propynlamine.HCl on DOPA, dopamine, norepinephrine, epinephrine and serotonin levels in mouse brain . . . 181
J. Frank Henderson and Irene G. Junga: Effects of purine antagonists on the metabolism of other purine antagonists . . . 187
Tibor Zsolnai: Versuche zur Entdeckung neuer Fungistatika—III. 8-Hydroxy-chinolin-Derivate, Nitroso-Verbindungen und Oxyme . . . 195
Walter D. Wosilait: The reduction of cytochrome *c* by reduced vitamin K_1 . . . 221

INGER ENANDER, ANDERS SUNDWALL and BO SÖRBO: Metabolic studies on N- methylpyridinium-2-aldoxime—I. The conversion to thiocyanate 226
INGER ENANDER, ANDERS SUNDWALL and BO SÖRBO: Metabolic studies on N-methylpyridinium-2-aldoxime—II. The conversion to N-methylpyridinium-2-nitrile 232
C. VAN DER MEER and P. W. VALKENBURG: The pharmacology of KCN as a prophylactic against radiation 237
A. M. ROTHSCHILD, I. VUGMAN and M. ROCHA E SILVA: Metabolic studies on the release of histamine by compound 48/80 in the rat diaphragm 248
S. H. NGAI, S. GINSBURG and R. L. KATZ: Action of thiamine and its analog on neuromuscular transmission in the cat 256
JAMES R. FOUTS, ROBERT L. DIXON and ROBERT W. SHULTICE: The metabolism of drugs by regenerating liver 265
L. LEVY and G. E. CRONHEIM: Blood levels and clearing activity of a synthetic heparinoid . . . 271

Short Communications:
H. Y. MELTZER: The effect of thiamine on monoamine oxidase 277
M. GOLDSTEIN and J. F. CONTRERA: Inhibition of dopamine β-oxidase by imipramine . . . 278
Book Review 280
Books Received 280

AUTHOR INDEX

AFFONSO, O. R., 78
AMER, S., 123
ANDRES, V. JR., 88
BARDI, U., 165
BER, A., 169
BERNHEIM, M. L. C., 59
BORETTI, G. 165
BOSTRÖM, H., 135
BRENK, H. A. S. VAN DEN, 35
BROPHY, E. M., 55
BUSCH, H., 123
CARTER, C. E., 17
CHAUBE, S., 17
CONTRERA, J. F., 77, 278
COURTNEY, K. D., 88
CRONHEIM, G. E., 271
DAVISON, C., 65
DI MARCO, A., 165
DIXON, R. L., 265
ELLMAN, G. L., 88
ENANDER, I., 226, 232
FIRSZT, D. C., 123
FISCHER, G. A., 75
FOUTS, J. R., 265
GINSBURG, S., 256
GOLDSTEIN, M., 77, 278
HARRIS, E. D. JR., 161
HENDERSON, J. F., 187
HOLZER, H., 1
HORITA, A., 47
HUGGINS, A. K., 151
JAMIESON, D., 35
JANSSEN, P. A. J., 81
JENEY, A., 23
JOHNSON, J. M., 100
JUNGA, I. G., 187
KARNOFSKY, D. A., 154
KATZ, R. L., 256
KOHEN, E., 123
KRÖGER, H., 1
LACON, C. R., 154
LAWFORD, D. J., 109
LEMIEUX, R. U., 31
LEVY, L., 271
LIPSEY, A., 123
MARKS, B. H., 96
MEER, C. VAN DER., 237
MELTZER, H. Y., 277
MITIDIERI, E., 78
NELSON, E., 31
NGAI, S. H., 256
O'REILLY, I., 31
PERRY, J. E., 181
RABINOVITZ, M., 100
ROCHA E SILVA, M., 248
ROTHSCHILD, A. M., 248
ROZMAN, R. S., 65
SCRIBA, P., 1
SHULTICE, R. W., 265
SINGER-ALTBEKER, R., 169
SMITH, D. L., 161
SMITH, M. J. H., 151
SMITH, P. K., 65
SOEP, H., 81
SÖRBO, B., 226, 232
SORGEN, R. W., 96
SPOREK, K. F., 31
STEGGLE, R. A., 151
SUNDWALL, A., 226, 232
SZENDREY, A., 23
VALKENBURG, P. W., 237
VIG, E., 23
VILLELA, G. G., 78
VUGMAN, I., 248
WEBER, L. J., 47
WENDEL, O. W., 55
WHITEHOUSE, M. W., 135
WIEGAND, R. G., 181
WILLS, E. D., 7
WOSILAIT, W. D., 221
ZSOLNAI, T., 195

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 1–6. Pergamon Press Ltd., Printed in Great Britain.

WIRKUNG CARCINOSTATISCHER ÄTHYLENIMIN-VERBINDUNGEN AUF DEN DPN-GEHALT THERAPIERESISTENER TUMOREN*

P. SCRIBA, H. KRÖGER und H. HOLZER

Physiologisch-Chemisches Institut der Universität
Freiburg im Breisgau

(*Received* 5 *December* 1960)

Abstract—Preliminary experiments with the ascites form of the relatively therapeutic-resistant DS-tumour demonstrated that the decrease in DPN content and the inhibition of glycolysis in the neoplastic cells under the influence of ethylenimine compounds were delayed, in comparison with the rapid changes observed in Ehrlich-ascites carcinomas. Extending these observations, the therapeutic effect of carcinostatic ethylenimine compounds was studied in rats bearing solid forms of either the Jensen sarcoma or the therapeutic-resistant DS-tumour. During the course of the experiments the DPN content of the tumours was followed. In the Jensen sarcoma the DPN content decreased sharply as early as the first day following administration of the carcinostatic, while the DS-tumour, in contrast, showed no clear change during the first six days. A cure was obtained in nine out of thirteen cases of the Jensen-sarcoma rats, while no cure was observed in six DS-tumour-bearing rats. These experiments further support our hypothesis, that carcinostatic ethylenimine compounds are therapeutically effective though they depress the DPN content in the tumour.

CARCINOSTATISCHE Äthylenimin-Verbindungen hemmen *in vitro* die anaerobe und aerobe Glykolyse von Ascitestumorzellen[3, 4] durch Senkung des Gehaltes an DPN.†[3, 5, 6] Auf Grund der von Warburg[7] gefundenen engen Beziehungen zwischen aerober Glykolyse und malignem Wachstum war zu vermuten, dass die Glykolysehemmung auch am Mechanismus der Heilung gewisser Tumoren im lebenden Tier entscheidend beteiligt sei. Hierfür sprechen die folgenden Beobachtungen:

(1) Nach intravenöser Verabreichung heilender Dosen von Äthyleniminverbindungen an Jensen-Sarkom tragende Ratten findet man ein weitgehend auf den Tumor beschränktes, d.h. gewebsspezifisches Absinken des DPN-Gehaltes, das dem makroskopisch sichtbaren Einschmelzen des Tumors vorausgeht.[8]

(2) Wird gleichzeitig mit heilenden Dosen von Äthylenimin-Verbindungen an Jensen-Sarkom-Ratten Nikotinsäureamid verabreicht, so bleibt das Absinken des DPN aus, und völlig parallel dazu wird die Heilung verhindert.[1, 8]

* Über die Ergebnisse der vorliegenden Arbeit wurde auszugsweise von P. Scriba beim IV Internationalen Biochemiekongress in Wien am 2 Sept. 1958[1] und am 27.1.1959 vor der Medizinischen Gesellschaft Freiburg im Breisgau[2] vorgetragen.

† Abkürzungen: DPN = Diphosphopyridinnucleotid; NSA = Nikotinsäureamid; TCE = Trichloressigsäure; DS-Tumor siehe Methoden, E 39 und A 139 siehe Präparate.

(3) Beim Vergleich der zur Heilung von Tumoren und zur Senkung des DPN-Gehaltes notwendigen Dosen verschiedener carcinostatischer Äthylenimin-Verbindungen findet man, dass auch hier Senkung des DPN-Gehaltes und Heilwirkung weitgehend parallel gehen.[9]

Zur weiteren Prüfung der Frage, wie weitgehend Heilwirkung und Wirkung auf den DPN-Gehalt von Tumoren sich entsprechen, wurde in der vorliegenden Arbeit die Wirkung von Äthylenimin-Verbindungen auf zwei verschiedene Tumoren, und zwar auf einen relativ therapieresistenten und einen der Therapie zugänglichen Tumor verglichen. Wir fanden in gutem Einklang mit den vorstehend aufgeführten früheren Versuchen wiederum Parallelität zwischen heilender und DPN senkender Wirkung.

METHODEN

1. *Tumoren*

Die Versuche wurden zum Teil an einem Ehrlich-Ascites-Carcinom durchgeführt, das uns von Herrn Professor Warburg freundlicherweise überlassen wurde und dessen Eigenschaften früher beschrieben wurden.[6, 10, 11] Herrn Professor Druckrey danken wir für das Jensen-Sarkom und für ein Carcino-Sarkom, das durch Dimethylaminostilbenfütterung (DS-Tumor) bei Ratten hervorgerufen wurde.[12] Der DS-Tumor wurde als solid, subkutan wachsender Tumor und als Ascitestumor untersucht. Es wurden Wistar-Ratten verwandt. Die Transplantation und die Technik der E 39-Behandlung wurden früher beschrieben.[8]

2. *Manometrie*

Es wurde die übliche Technik der Manometrie mit der Warburg-Apparatur angewandt (cf. Holzer *et al.*[4]). Jeweils 30–50 mm^3 Zellen pro ml Krebs-Ringer-Bicarbonatlösung wurden bei 37 °C im Warburg-Gefäss inkubiert. Das pH wurde durch CO_2 Begasung auf 6,0 eingestellt und Glucose in 0,025 M Endkonzentration zugesetzt.

3. *DPN-Bestimmung*

Die Aufarbeitung der Zellsuspensionen und der soliden Tumoren sowie die DPN-Bestimmung mit dem optischen Test nach Warburg wurden früher beschrieben.[6, 8, 10, 11]

PRÄPARATE

Von der Firma Bayer-Leverkusen wurden uns freundlicherweise die Substanzen E 39 (2,5-Di-*n*-propoxy-3,6-bisäthyleniminobenzochinon-1,4) und A 139 (2,5-Dimethoxy-äthoxy-3,6-bisäthylenimino-benzochinon-1,4) zur Verfügung gestellt. Wir möchten auch an dieser Stelle den Herren Professor Dr. G. Domagk und Dr. S. Petersen für die Überlassung der Präparate danken.

ERGEBNISSE

1. *Versuche mit Ascites-Tumoren*

In Tabelle 1 sind Ergebnisse von manometrischen Glykolysemessungen und von DPN-Bestimmungen in Zellen der Ascitesform des von Druckrey *et al.*[12] als besonders therapieresistent beschriebenen DS-Tumors zusammengestellt. Aus den in der untersten Zeile angegebenen Glykolyse-Daten sieht man, dass die hier untersuchten Zellen sich im Prinzip genau so verhalten, wie die früher von uns untersuchten Zellen des

Ehrlichschen Ascites-Carcinoms:[6, 10] Zusatz der carcinostatischen Äthylenimin-Verbindung A 139 bewirkt eine kräftige Hemmung der Glykolyse, gleichzeitiger Zusatz von Nikotinsäureamid hebt, wie die letzte Kolonne der Tabelle zeigt, die Glykolysehemmung auf. Betrachtet man jedoch die Ergebnisse der DPN-Analysen, so sieht man, dass der Abfall des DPN-Gehaltes sehr spät einsetzt. Nach 80 min Inkubation mit A 139 ist der DPN-Gehalt erst um ca. 40% gesunken.

TABELLE 1. WIRKUNG VON NSA AUF ANAEROBE GLYKOLYSE UND DPN-GEHALT VON ASCITESZELLEN DES DS-TUMORS BEI INKUBATION MIT UND OHNE A 139

Manometrie und DPN-Bestimmung sind unter Methoden beschrieben. Pro Ansatz (Gesamtvolumen 6 ml) wurden 162 mm^3 Zellen eingesetzt. Die Konzentration des A 139 betrug $0{,}18 \times 10^{-3}$ M (= 6 mg %), die des NSA 0,016 M.

	Behandlung			
	Kontrolle	A139	NSA	A139 + NSA
$\frac{\mu\text{Mole DPN}}{mm^3 \text{ Zellen}} \times 10^4$ nach 80 min Inkubation*	2,73	1,67	3,18	2,38
$\frac{\mu\text{Mole DPN}}{mm^3 \text{ Zellen}} \times 10^4$ nach 240 min Inkubation*	1,75	<0,2	2,45	1,67
mm^3 CO_2 pro mm^3 Zellen von der 210–240 min nach Glucosezusatz	0,52	0,11	0,51	0,45

* Es handelt sich bei den beiden Versuchsreihen zur DPN-Bestimmung um verschiedene Versuche.

Es war zu vermuten, dass die durch den langsamen DPN-Abfall bewirkte sehr spät einsetzende Glykolysehemmung mit den besonderen Resistenzeigenschaften des hier untersuchten Ascites-DS-Tumors zusammenhängt. Deshalb wurde dieser mit dem Ehrlichschen Ascites-Carcinom verglichen. In Abb. 1 sind Glykolysewerte und der DPN-Gehalt der Zellen nach Abschluss der Glykolysemessungen (rechteckig eingerahmte Werte) eingetragen. Während im Ehrlichschen Ascites-Carcinom die Glykolyse rasch abfällt und nach etwa 60 min vollständig gehemmt ist, setzt eine Hemmung der Glykolyse beim DS-Tumor erst nach ca. 100 min ein. Vollständige Hemmung ist nach etwa 240 min erreicht. Zu diesem Zeitpunkt ist beim DS-Tumor ebenso wie beim Ehrlich-Ascites-Carcinom bei völliger Glykolysehemmung der DPN-Gehalt auf einen mit unserer Methode nicht mehr messbaren Wert abgesunken. Der besonders therapieresistente DS-Tumor zeichnet sich demnach auch durch Unempfindlichkeit gegen den DPN-senkenden und glykolysehemmenden Einfluss von Äthyleniminverbindungen aus.

2. *Versuche mit soliden Tumoren*

Um die Therapieresistenz, d.h. das Eintreten bzw. Ausbleiben von Heilerfolgen, in Zahlen angeben zu können, wurden die in Tabelle 2 niedergelegten Versuche mit den soliden Formen des DS-Tumors und des Jensen-Sarkoms durchgeführt. Der DS-Tumor war gegen die in diesen Versuchen eingesetzte Äthylenimin-Verbindung

E 39 weitgehend resistent: es wurden keine Heilungen, sondern lediglich Lebensverlängerungen beobachtet. Beim Jensen-Sarkom wurden 9 von 13 Tieren geheilt (das Ausbleiben eines Rezidivs innerhalb einer fünfmonatigen Nachbeobachtung wurde von uns als Heilung bezeichnet). Die Ergebnisse von DPN-Bestimmungen in den Tumoren völlig parallel geführter Tiere sind in Abb. 2 eingetragen. Während

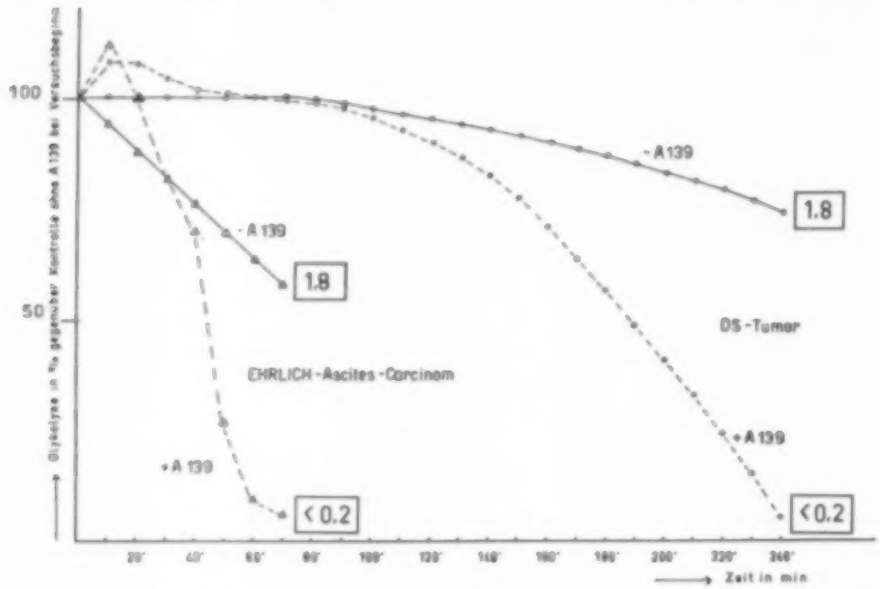


Abb. 1. Anaerobe Glykolyse und DPN-Gehalt von Ehrlich-Ascites-Carcinom und Ascitesform des DS-Tumors. Endkonzentration an A 139: $0{,}18 \times 10^{-3}$ M. Manometrie siehe Methoden. Nach der letzten in die Abbildung eingetragenen manometrischen Ablesung wurden die Zellen mit TCE abgetötet und aufgeschlossen. Die Ergebnisse der mit dem klarzentrifugierten Überstand durchgeführten DPN-Bestimmungen in μMole DPN $\times 10^4$ pro mm³ Zellen sind rechteckig eingerahmt in die Abbildung eingetragen.

Tabelle 2. Vergleich der Therapieempfindlichkeit gegenüber E 39 von Jensen-Sarkom und solidem DS-Tumor bei Wistar-Ratten

Die Technik der Beimpfung und die Behandlung der Tiere wurden früher beschrieben.[5]

Tumor	Zahl der mit 3,1 mg E 39 pro kg behandelten Tiere	Geheilt (5 Monate Nachbeobachtung)	Gestorben
Jensen-Sarkom	13	9	4*
DS-Tumor	6	0	6*

* Die behandelten Tiere überlebten die unbehandelten Kontrolltiere um 8 Tage (Jensen-Sarkom) bzw. 27 Tage (DS-Tumor).

beim Jensen-Sarkom der von uns schon früher mitgeteilte[8] rasche Abfall des DPN-Gehaltes eintritt, nimmt im DS-Tumor der DPN-Gehalt in den ersten Tagen nach Verabreichung von E 39 nicht ab. Ein Vergleich der in Tabelle 2 angegebenen Heilungserfolge mit dem in Abb. 2 niedergelegten Verhalten des DPN-Gehaltes

demonstriert die Parallelität von Heilwirkung und Wirkung auf den DPN-Gehalt der Tumoren.

ZUSAMMENFASSUNG

In orientierenden Versuchen mit der Ascites-Form des relativ therapieresistenten DS-Tumors wurde gefunden, das der DPN-Abfall und die Glykolysehemmung durch Äthylenimin-Verbindungen im Vergleich zum Ehrlich-Ascites-Carcinom verzögert

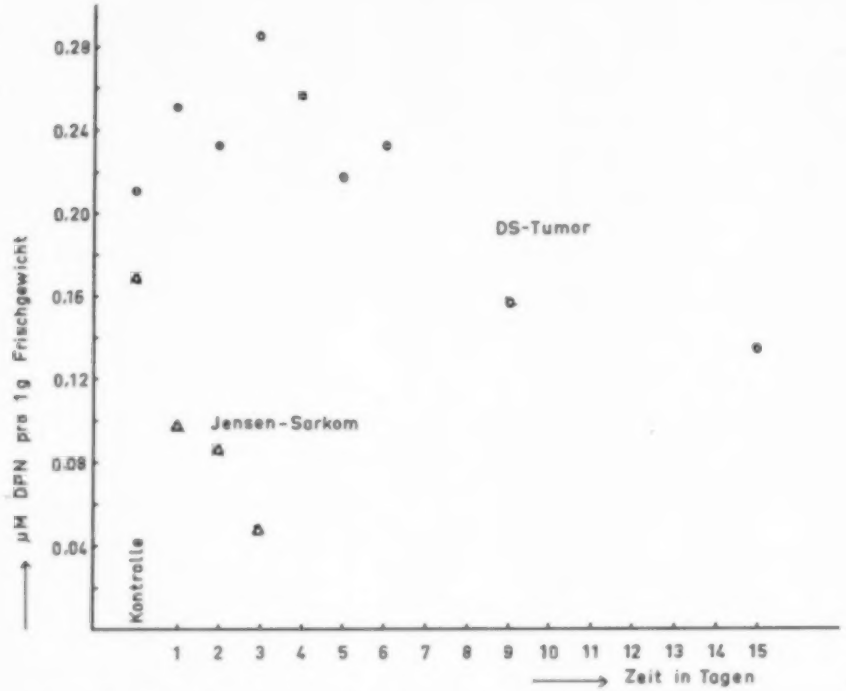


ABB. 2. DPN-Gehalt in Jensen-Sarkom und DS-Tumor (solide Form) nach einmaliger Verabreichung von 3,1 mg E 39/kg Körpergewicht. Die als "Kontrolle" (nach 0 Tagen) eingetragenen Werte wurden von Tieren gemessen, die nicht mit E 39 behandelt wurden.

auftreten. Aufbauend auf diesen Befund wurde die Heilwirkung carcinostatischer Äthylenimin-Chinon-Verbindungen bei Jensen-Sarkom- bzw. den therapieresistenten DS-Tumor-tragenden Ratten studiert. Parallel dazu wurde der DPN-Gehalt in den Tumoren gemessen. Im Jensen-Sarkom fällt der DPN-Gehalt bereits einen Tag nach Verabreichung des Carcinostaticums stark ab, während im DS-Tumor in den ersten 6 Tagen keine deutliche Veränderung zu beobachten ist. Dementsprechend wurde bei den Jensen-Sarkom tragenden Tieren in 9 von 13 Fällen eine Heilung beobachtet, während bei keinem der 6 DS-Tumor tragenden Tiere Heilung eintrat. Die vorgelegten Versuche sind eine weitere Stütze für unsere Annahme, dass carcinostatische Äthylenimin-Verbindungen durch Senkung des DPN-Gehaltes in Tumoren deren Heilung bewirken.

Anerkennungen—Wir danken der Deutschen Forschungsgemeinschaft und dem Bundesministerium für Atomkernenergie und Wasserwirtschaft für Sachbeihilfen.

LITERATUR

1. P. SCRIBA, H. KRÖGER und H. HOLZER, IV Internationaler Biochemie Kongress, Wien, 1–6 September 1958, Sektion **14**, 9. *International Abstracts of Biological Sciences*, 1958.
2. P. SCRIBA, *Klin. Wschr.* **37**, 678 (1959).
3. I. M. ROITT, *Biochem. J.* **63**, 300 (1956).
4. H. HOLZER, G. SEDLMAYR und A. KEMNITZ, *Biochem. Z.* **328**, 163 (1956).
5. H. HOLZER und G. SEDLMAYR, *Ber. ges. Physiol.* **189**, 120 (1957).
6. H. HOLZER, P. GLOGNER und G. SEDLMAYR, *Biochem. Z.* **330**, 59 (1958).
7. O. WARBURG, *Über den Stoffwechsel der Tumoren*. Julius Springer, Berlin (1926).
8. H. HOLZER und H. KRÖGER, *Biochem. Z.* **330**, 579 (1958).
9. H. KRÖGER, B. ULRICH und H. HOLZER, *Arzneimittel-Forsch.* **9**, 598 (1959).
10. P. SCRIBA, S. SCHNEIDER und H. HOLZER, *Z. Krebsforsch.* **63**, 547 (1960).
11. P. SCRIBA, Dissertation, Medizinische Fakultät der Universität Freiburg i. Br. (1959).
12. H. DRUCKREY, D. SCHMÄHL, P. DANNEBERG, K. KAISER, H. A. NIEPER, H. W. LO, R. MECKE JR., J. VON EINEM und W. DISCHLER, *Arzneimittel-Forsch.* **6**, 539 (1956).

Vol. 7
1961

Biochemical Pharmacology 1961, Vol. 7, pp. 7–16. Pergamon Press Ltd., Printed in Great Britain.

EFFECT OF UNSATURATED FATTY ACIDS AND THEIR PEROXIDES ON ENZYMES

E. D. WILLS

Department of Biochemistry, Medical College of St. Bartholomew's Hospital, London, England.

(*Received* 19 *August* 1960)

Abstract—Many enzymes of various types, e.g. urease, glyoxalase and papain, have been found to be inhibited by emulsions of oxidised unsaturated fatty acids such as linoleic or linolenic. Some enzymes, such as catalase and D-amino acid oxidase, were unaffected. In most cases, the extent of the inhibition was dependent on the peroxide value of the emulsion, and on the time of contact of enzyme and emulsion. In general, —SH enzymes were found to be more readily inhibited than enzymes not possessing —SH groups and to be protected by sulphydryl compounds, such as glutathione.

INTRODUCTION

CERTAIN unsaturated fatty acids such as linoleic, linolenic and arachidonic have been known for many years to be dietary essentials for mammals. The precise function of these acids is not yet clear, although they are known to be combined as phospholipids and form part of essential cell components such as the cell membranes.

During the past few years, interest in these fatty acids has been stimulated for two main reasons; firstly on account of the relationship of unsaturated fatty acids to cholesterol metabolism,[1] and secondly on account of the fact that peroxides formed from unsaturated fatty acids may be responsible for radiation damage.[2]

Several investigators have shown that there is a connexion between peroxide formation and radiation damage to mammals. Thus Horgan *et al.*[3] found that peroxides formed from linoleic acid were toxic when injected into mice. Initially, these toxic effects were considered to be similar to the effects of radiation, but toxic effects resulting from the administration of fatty acid peroxides have subsequently been shown to develop much more rapidly.[4] However, Muset, Esteve and Mateu,[5] found that lipoxidase, which oxidises unsaturated fatty acids to peroxides, was toxic when injected into or fed to mice. Many of the lipoxidase effects were found to be very similar to radiation effects, and these workers classed lipoxidase as radiomimetic. Other organic peroxides have also been shown to be radiomimetic.[6]

The toxicity of fatty acid peroxides must be the result of some biochemical damage, but as yet few studies have been made of the effect of these peroxides on vital cellular processes. Bernheim, Wilbur and Kenaston[7] have shown, however, that fatty acid peroxides inhibit some oxidative enzymes and Dubouloz and Fondarai[8] found that ethyl oleate peroxide was capable of oxidising the —SH groups of proteins to —S—S— and to further oxidation products. More recently, Tappel and Zalkin[9] found that liver mitochondria took up oxygen in the absence of added substrate and

that this oxygen uptake resulted in the formation of lipid peroxides. During the formation of these peroxides there was a concomitant destruction of some of the mitochondrial enzymes, e.g. succinic oxidase and DPNH-cytochrome c reductase.

The effect of emulsions of oxidised unsaturated fatty acids has now been examined on a wide variety of enzymes. It has been found that peroxide inhibition is not confined to oxidative enzymes and that several enzymes of other classes, e.g. hydrolytic enzymes, are inhibited. Furthermore, certain oxidative enzymes have been found to be resistant to peroxide inhibition. In general, sulphydryl enzymes have been found to be much more strongly inhibited by peroxides than enzymes not possessing —SH groups.

A preliminary account of some of this work has already been published.[10]

MATERIALS AND METHODS

Fatty Acids. Oleic acid, palmitic acid and stearic acid were pure samples obtained from B.D.H. Ltd.. Pure linoleic acid was obtained from the California Research Foundation. Pure linolenic acid was obtained from Mann Research Laboratories, New York, U.S.A..

Mixed linseed acids. These were prepared by the hydrolysis of linseed oil using the method described by Hilditch.[11] An analysis of these acids on a Gas Chromatogram showed that the mixture contained approximately 45% linolenic acid, 20% linoleic acid, 12% oleic acid and 23% saturated acids. The iodine number was found to be 188. A value of 280 was taken for the average molecular weight.

Fatty acid emulsions. For the majority of experiments the fatty acid was dissolved in ethanol to give an 0·1 M solution. 1 ml of this solution was then added to 9 ml 0·05 M phosphate buffer (pH 7·4) and the mixture was quickly shaken to produce a stable emulsion containing 10% ethanol and 0·01 M fatty acids. When weaker concentrations of fatty acid were used, a clear solution was obtained. Control experiments using buffer containing 10% ethanol were always carried out when testing the effect of these emulsions on enzymes. For some experiments, emulsions were prepared by dissolving the required quantity of fatty acid in an equivalent amount of ammonium hydroxide or sodium hydroxide.

Enzyme preparations and activity measurements. The methods of preparation and determination of the enzymes used have been previously described by Wills.[12, 13, 14, 15]

RESULTS

Comparison of the effects of saturated and unsaturated fatty acids on enzymes

The effect of emulsions containing 2×10^{-3} M palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, or mixed linseed acids in 0·05 M pH 7·4 phosphate buffer was examined on 18 different enzymes. In each case the enzyme was incubated with the emulsion for 20 min at 37° before determining the activity.

Under these conditions the enzymes examined were found to be divisible into four groups:

A. Those unaffected by either saturated or unsaturated fatty acids;

B. Those inhibited by saturated and unsaturated fatty acids to an approximately equal extent;

C. Those unaffected by saturated fatty acids but inhibited by unsaturated fatty acids;

Vol. 7
1961

D. Those inhibited to a greater extent by unsaturated than by saturated fatty acids.

The enzymes tested are shown listed in their various groups in Table 1. In Table 2 specific figures are given for the inhibition of representative members of each group by equivalent concentrations of palmitic and linoleic acid emulsions. Stearic acid emulsions were always found to behave exactly as those of palmitic acid and emulsions of oleic acid resembled those of linoleic acid but their inhibitory action was always very much weaker.

TABLE 1. COMPARISON OF THE EFFECTS OF PALMITIC ACID EMULSION (2×10^{-3} M) AND LINOLEIC ACID EMULSION (2×10^{-3} M) ON SOME ENZYMES

Group	Enzymes		Palmitic acid emulsion	Linoleic acid emulsion	Comment
A	Xanthine oxidase Tyrosinase	Catalase D-Amino acid oxidase	No effect	No effect	
B	Cholinesterase Serum esterase Lipase	Invertase Pepsin	Inhibited	Inhibited	Inhibition by two acids approximately equal
C	Glyoxalase (−SH) Choline oxidase (—SH)	β-amylase (−SH) Liver respiration (—SH)	No effect	Inhibited	
D	Succinic oxidase (—SH) Cytochrome oxidase	Trypsin Papain (—SH) Urease (—SH)	Inhibited	Inhibited	Inhibition by linoleic acid much greater than by palmitic acid

TABLE 2. A COMPARISON OF THE EFFECTS OF PALMITIC ACID EMULSION (2×10^{-3} M) AND LINOLEIC ACID EMULSION (2×10^{-3} M) ON REPRESENTATIVE ENZYMES OF GROUPS A, B, C AND D

Group	Enzyme	Inhibition, per cent	
		Palmitic acid (2×10^{-3} M)	Linoleic acid (2×10^{-3} M)
A	Catalase	0	0
B	Serum esterase	32	32
C	Glyoxalase	0	83
D	Papain	21	86

Group classifications are described in Table 1 and the text.

The pattern of the inhibition of enzymes of Group D was changed when dilute, e.g. 4×10^{-4} M, emulsions of palmitic acid and linoleic acid were compared. At this concentration palmitic acid had virtually no effect, whilst linoleic acid was strongly inhibitory (Fig. 1).

The effect of emulsions of unsaturated fatty acids, such as linoleic or linolenic, on the enzymes of groups C and D was found to be mainly dependent on two factors: firstly, on the state of oxidation, or peroxide value of the fatty acid; and secondly, on the duration of contact of emulsion and enzyme. These effects are discussed in a subsequent section of this paper.

Enzyme inhibition by saturated fatty acids such as was found with the enzymes of group B is clearly unconnected with double bonds or their oxidation. It is an unspecific effect and possibly results from the affinity of the negative charges of the fatty acids for the positive charges on the protein. It has been previously discussed by Putnam[16] and Wills.[12]

On the other hand, enzymes of groups C and D are inhibited to a much greater extent by linoleic acid than by palmitic acid, and this effect must be directly related to the presence of unsaturated linkages or the peroxides formed at these bonds.

Bernheim *et al.*[7] have already shown that certain oxidative enzymes are inhibited by unsaturated fatty acids. It will be observed from Table 1 however, that the inhibitory effect of unsaturated fatty acids is not confined to oxidative enzymes, and that several enzymes of other classes, e.g. urease and glyoxalase are also inhibited.

A more significant property of the enzymes included in Groups C and D is that, with the exception of trypsin and cytochrome oxidase, they all possess essential —SH groups. Furthermore, all enzymes tested possessing essential —SH groups were inhibited by linoleic acid.

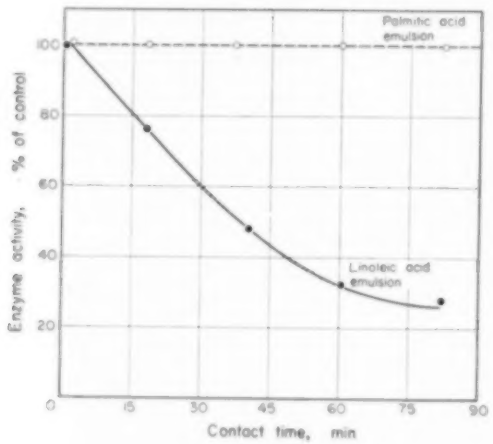


FIG. 1. A comparison of the effects of palmitic acid emulsion (4×10^{-4} M) and linoleic acid emulsion (4×10^{-4} M) on succinic oxidase 37 ° when the time of contact of emulsion and enzyme is varied.

Effect of contact time and temperature on enzyme inhibition

Most experiments described in this paper were carried out using a contact period of emulsion and enzyme of 20 min and at 37°. Inhibition of all enzymes of Groups C and D was found to increase with increase of the contact time. In Fig. 1 a comparison is made between the effects of palmitic and linoleic acids in equivalent concentrations

on succinic oxidase. It will be observed from Fig. 1 that inhibition of the enzyme increases progressively with time of contact.

For certain enzymes the temperature of the incubation was also found to be important. Thus the inhibition of urease by linseed acid emulsion was found to be much greater after 4 hr incubation at 37 ° than after 4 hr incubation at 16 °.

Relation between the extent of oxidation of the fatty acid emulsion and enzyme inhibition

Unsaturated fatty acids readily take up oxygen to give peroxides. These are probably hydroperoxides (—OOH) formed on a carbon atom adjacent to the double bond.[17]

Emulsions containing 10^{-2} M linoleic acid, linolenic acid or mixed linseed acids were oxidised by shaking in air at 37° for periods which were varied between 3 hr and 48 hr. Catalysts such as haemin which speed the rate of autoxidation were added to some emulsions. The extent of the oxidation was measured either (a) by a determination of the peroxide value by the ferric thiocyanate method[18] or (b) for emulsions of linolenic acid or mixed linseed acids by the thiobarbituric acid colorimetric method.[19]

A series of emulsions of unsaturated fatty acids of different peroxide values were tested on urease, succinic oxidase and papain. For each experiment the oxidised emulsion was incubated with the enzyme for 20 min at 37° before determination of the enzyme activity. Whichever method was used for the determination of the peroxidation, enzyme inhibition was always found to increase progressively in parallel with the increase of peroxide value of the emulsion. Results for urease and papain are shown in Figs. 2 and 3. Inhibition was found to be dependent on the time of contact

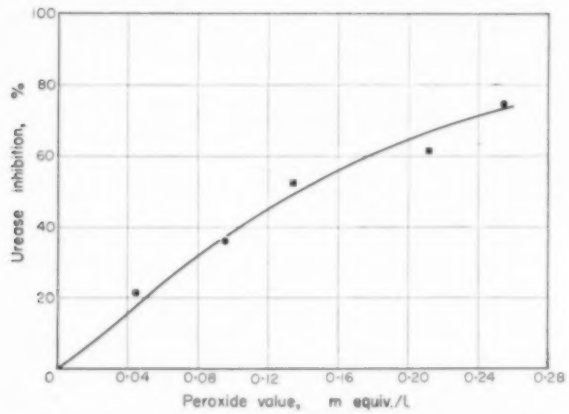


FIG. 2. Relation between the peroxide value of a mixed linseed acid emulsion (10^{-3} M) and urease inhibition. Peroxide values determined by the thiobarbituric acid method.

of emulsion and enzyme as in other experiments, but the fatty acid emulsion of higher peroxide value inhibited the enzyme much more strongly than an emulsion of low peroxide value when each was incubated with the enzyme for the same time period (Fig. 4).

Effect of ultraviolet light on mixtures of fatty acid emulsions and enzymes

Ultraviolet light catalyses the formation of peroxides from unsaturated fatty acids[20] and therefore irradiation of enzymes in presence of unsaturated fatty acids

would be expected to cause significant inactivation of enzymes sensitive to these peroxides.

In order to investigate the effect of ultraviolet light, open dishes of solutions were irradiated 30 cm from a Hanovia u.v. lamp. In each experiment the following systems were irradiated: (a) the enzyme alone, (b) the enzyme + palmitic acid emulsion (10^{-3} M or 4×10^{-4} M), (c) enzyme + linoleic acid or mixed linseed acid emulsion

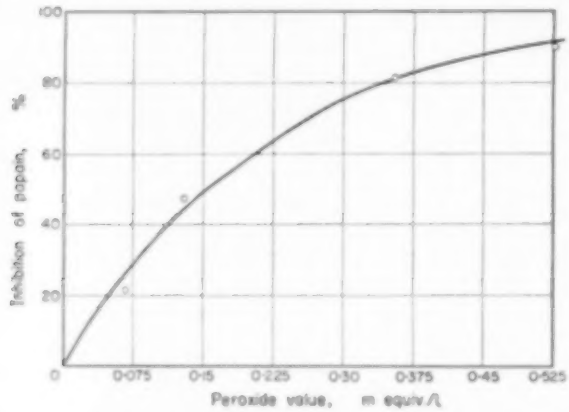


FIG. 3. Relation between the peroxide value of a linoleic acid emulsion (10^{-3} M) and inhibition of papain. Peroxide values determined by the ferric thiocyanate method.

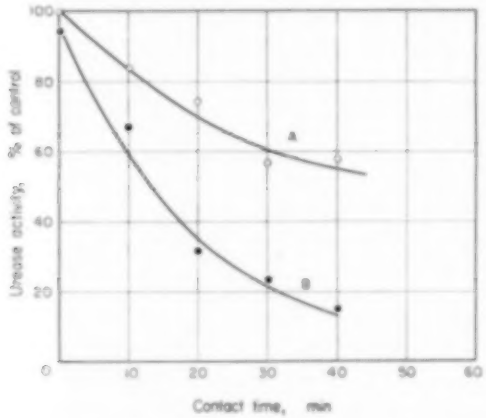


FIG. 4. Relation between the peroxide value of a linseed acid emulsion (10^{-3} M) and contact time on the inhibition of urease.
Curve A—Peroxide value = 0·045 mequiv/l.
Curve B—Peroxide value = 0·21 mequiv/l.

(10^{-3} M or 4×10^{-4} M), (d) linoleic acid or mixed linseed acid emulsions (10^{-3} M or 4×10^{-4} M), (e) palmitic acid emulsion (10^{-3} M or 4×10^{-4} M). After the irradiation the activity of the untreated enzyme was compared with the enzyme irradiated under conditions (a), (b) or (c). In addition, irradiated linoleic acid and irradiated palmitic acid emulsions were tested on the untreated enzyme.

Succinic oxidase, β-amylase, and invertase were irradiated under these conditions for periods which were varied between 2 hr and 6 hr. Succinic oxidase, after being treated with u.v. for 2 hr was 14 per cent inhibited, but the inhibition increased to 63 per cent if 4×10^{-4} M linoleic acid emulsion was present during the irradiation. Irradiated linoleic acid added to the untreated enzyme caused only 40 per cent inhibition after 30 min contact. Palmitic acid emulsion (4×10^{-4} M) on the other hand, did not alter the effect of u.v., the enzyme being 14 per cent inhibited as in absence of emulsion. The inhibition of β-amylase was increased from 4 per cent after 2 hr u.v. irradiation to 20 per cent when linoleic acid emulsion (10^{-3} M) was added before irradiation. On the other hand, invertase which had previously been shown not to be inhibited by fatty acid peroxides, was unaffected by irradiation in presence of 10^{-3} M unsaturated fatty acids.

Effect of lipoxidase

Lipoxidase catalyses the oxidation of unsaturated fatty acids such as linoleic or linolenic to form peroxides at their double bonds.

The effect of enzymically produced peroxides was investigated by incubating lipoxidase solution (0·5 mg/ml of a pure Sigma preparation) with $6{\cdot}6 \times 10^{-4}$ M linoleic acid emulsion for 20 min when oxygen uptake ceased. The resulting oxidised emulsion when tested on urease caused 77 per cent inhibition, whilst the untreated emulsion in the same concentration caused only 43 per cent inhibition. The effect of lipoxidase itself on urease was negligible. Inactivation of the urease was greater if it was incubated with lipoxidase actively oxidising the unsaturated fatty acids. Thus urease was 30 per cent inhibited by $6{\cdot}6 \times 10^{-4}$ M linseed acid emulsion added to the enzyme after oxidation was complete, but the inhibition was increased to 88 per cent if the lipoxidase oxidised the emulstion in presence of urease. Incubation of urease with lipoxidase alone for a 20-min period was found to have a negligible effect on urease activity.

Protection of enzymes against inhibition by unsaturated fatty acids

Experiments described in this paper have shown that enzymes possessing —SH groups are most sensitive to inhibition by peroxides. If the —SH groups are vulnerable sites for enzymic inactivation by peroxides, protection is likely to be afforded and the inhibited enzymes possibly reactivated by the addition of sulphydryl compounds such as cysteine or glutathione.

TABLE 3. EFFECT OF CYSTEINE ON THE INHIBITION OF UREASE BY LINSEED ACID EMULSION

Time of contact before cysteine addition (min)	Inhibition (per cent)
No cysteine	69
0	25
5	33
30	67

Cysteine (10^{-2} M) added after urease had been in contact with linseed acid emulsion ($3{\cdot}3 \times 10^{-3}$ M) for time specified above.

The effect of cysteine (10^{-2} M) was examined, either by adding it to the urease before addition of the fatty acid emulsion ($3{\cdot}3 \times 10^{-3}$ M linseed acid emulsion) or by adding it a short period after. Results are shown in Table 3. As will be seen from this

table, cysteine gave partial protection of the enzyme and partial reactivation if the period of contact of emulsion and enzyme was not prolonged. After longer contact periods no reversal was possible.

Cysteine (10^{-2} M) was also found to protect succinic oxidase, reducing the inhibition by $3{\cdot}3 \times 10^{-4}$ M mixed linseed acids from 80 per cent to 36 per cent. Papain was also protected, 10^{-2} M cysteine reducing the inhibition by 10^{-3} M mixed linseed acid emulsion from 65 per cent to 12 per cent.

DISCUSSION

The inhibition of enzymes by fatty acids described in this work clearly falls into two categories: (i) that which is unspecific, being caused by saturated and unsaturated fatty acids alike, and (ii) that which is specifically caused by unsaturated fatty acids.

Enzyme inhibition of type (i) has been previously described by Peck[21] who found that trypsin was inhibited by sodium salts of saturated fatty acids and by Wills[12] who found that the related anionic detergents such as sodium dodecyl sulphate powerfully inhibited a large variety of enzymes. Many of the enzymes tested were strongly inhibited by anionic detergents when the pH of the solution was acid. This inhibition is probably the result of combination of the basic groups of the protein with the acidic detergent groups. So far as possible, this effect was avoided in the present work by using a pH of 7·4 for most experiments.

In view of these findings it may be concluded that inhibition of enzymes of group B (enzymes which are inhibited by saturated and unsaturated fatty acids to an approximately equal extent) is probably a non-specific effect resulting from the combination of negatively charged fatty acids and basic protein groups which leads to the denaturation of the enzyme.

The inhibition of enzymes of group D by unsaturated fatty acids is likely to be the result of two distinct effects. Firstly, a result of combination of the negative charged fatty acids with the basic protein groups, and secondly, a result of the presence of unsaturated bonds in the fatty acid. However, when the inhibitory effects of less concentrated emulsions (e.g. 4×10^{-4} M) of saturated and unsaturated fatty acids were compared it was frequently found that only unsaturated fatty acids caused inhibition (Fig. 1).

Enzymes of group C are not affected at pH 7·4 by negative charges on the fatty acids and inhibition in these cases is solely a result of the presence of double bonds or peroxides in the unsaturated fatty acids.

When first prepared, emulsions of unsaturated fatty acids gave small but measurable peroxide values. Such emulsions caused some enzyme inactivation but this was found to increase progressively as the period of incubation of the emulsion with the enzyme was increased.

When unsaturated fatty acids are oxidised, peroxides, probably hydroperoxides, are formed at the CH groups adjacent to the double bonds, a seift of o double bond occurring during the oxidation.[17]

Inhibition of the enzymes of groups C and D by unsaturated fatty acids may be explained on the basis that it is these peroxides which are responsible. Evidence for this opinion is summarised in Figs. 2 and 3, where it is shown that the enzyme inhibition increases with increase of peroxide value. Emulsions of low peroxide value were found to be much less inhibitory than emulsions of higher value but the inhibition increased

Vol. 7
1961

with the time of contact with the enzyme. This was presumably the result of a slow development of peroxide. When peroxides were preformed the inhibitory effect was developed much more rapidly (Fig. 4). Experiments using lipoxidase and ultraviolet light showed that these peroxides were often more toxic if formed in presence of the enzyme than if added to the enzyme after formation. The reason for this is not clear but it is possible that free radicals generated during peroxide formation are more toxic to the enzyme than the peroxides themselves. This phenomenon may be of considerable importance in relation to possible *in vivo* actions of peroxides which are discussed later.

An inspection of the enzymes listed in Table 1 will indicate that —SH enzymes are most readily inactivated by linoleic acid. In fact, all enzymes tested which are known to possess essential —SH groups were inactivated by linoleic acid, and trypsin and cytochrome oxidase are the only enzymes of groups C and D which are not normally regarded as —SH enzymes. The —SH groups of enzymes are likely to be vital targets for peroxide inhibition on account of (a), the nature of the enzymes inhibited and (b) the fact that —SH compounds such as cysteine or glutathione are protective. After short periods of contact, limited reversal of inactivated sulphydryl enzymes by —SH compounds was obtained but after longer contact periods, no reversal was possible. Furthermore, it has been previously shown by Dubouloz and Fondarai[8] that the —SH groups of sulphydryl proteins are oxidised to —S—S— and further oxidation products by fatty acid peroxides. The results of the experiments using —SH enzymes may be interpreted as indicating that there is an initial oxidation of —SH to —S—S— but that further changes are produced in the molecule. Simple oxidation of —SH to —S—S— cannot be the only reaction involved since this would be reversed by cysteine or glutathione. In this respect, the inactivation of sulphydryl enzymes by fatty acid peroxides is closely parallel to inactivation of this class of enzyme by organic peroxides as described by Wills.[15]

These experiments have confirmed the observations of Bernheim *et al.*[7] that oxidation enzymes are inhibited but it has been found that the inhibitory effect is not confined to this class of enzyme, since several other classes of enzyme, e.g. hydrolases are inhibited. As discussed above, it is considered that the most sensitive enzymes are —SH and the fact that several are oxidative is incidental. All the oxidative enzymes found to be inhibited by Bernheim *et al.*[7] are normally regarded as —SH enzymes. Tappel and Zalkin[9] have also shown recently that mitochondria took up oxygen forming peroxides, several oxidative enzymes being destroyed in the process.

The true role of the inhibitory effect of peroxides of fatty acids *in vivo* is not easy to establish, but nevertheless, it is possible that it may be of considerable importance in metabolic control. The oxidation of fatty acids to peroxides can be powerfully catalysed by iron compounds such as haemin in very low concentrations.[22] These peroxides may then slow a vital metabolic path by inhibiting a vital —SH enzyme. Located in important membranes around or within the cell, probably as constituents of phosphatides, it appears possible that unsaturated fatty acids may be well sited to exert an important metabolic control.

It is also possible that the oxidation of unsaturated fatty acids may be connected with the effects of radiation. Relationships between peroxides and radiation have been established from several avenues of research; firstly, it has been shown that peroxides are formed in irradiated animals,[4] secondly, that some of the toxic effects of injected

peroxides resemble radiation effects,[3] and thirdly, that the injection of lipoxidase causes certain radiomimetic effects.[5]

It is therefore conceivable that X-rays may stimulate the development of the peroxides from unsaturated fatty acids at vital sites in the cell. Certain well established facts of radiation damage are in accordance with this view, the dependence on oxygen, the temperature effect whereby radiation effects are much less at lower temperatures, and the delayed effect of radiation damage which is also temperature dependent. These peroxides in their turn, developing slowly, may well cause damage to some vital —SH protein, possibly an enzyme. In order to attempt to establish the validity of this theory, experiments have been started to study the irradiation of unsaturated fatty acids under various conditions and in the presence of sulphydryl enzymes. Preliminary results have shown that —SH enzymes may undergo considerable destruction when irradiated in presence of unsaturated fatty acids.[23]

Acknowledgements—The author wishes to express his thanks to Professor A. Wormall for advice and interest in this work and to Miss S. E. Lewis for her assistance with some of the experiments.

REFERENCES

1. H. M. SINCLAIR (Editor) *Essential Fatty Acids* 4th International Conference on Biochemical Problems of Lipids. Butterworth, London (1958).
2. M. HAISSINSKY (Editor) *Les Peroxydes Organiques en Radiobiologie*, Masson et Cie, Paris (1958).
3. V. J. HORGAN, J. ST. L. PHILPOT, B. W. PORTER and D. B. RODDYN. *Biochem. J.*, **67**, 551 (1957).
4. J. ST. L. PHILPOT and D. B. RODDYN, *Int. J. Rad. Biol.* **1**, 372 (1959).
5. P. G. MUSET, J. M. ESTEVE, and J. V. MATEU, *Nature, Lond.* **184**, 1506 (1959).
6. R. LATARJET, *Ciba Foundation Symposium on Ionising Radiations and Cell Metabolism*, p. 275 (1956).
7. F. BERNHEIM, K. M. WILBUR and C. B. KENASTON, *Arch. Biochem.* **38**, 177 (1952).
8. P. DUBOULOZ and J. FONDARAI, *Bull. Soc. Chim. Biol.* **35**, 819 (1953).
9. A. L. TAPPEL and H. ZALKIN, *Arch. Biochem.* **80**, 326 (1959).
10. E. D. WILLS, *Abstracts, Fourth Int. Congress of Biochemistry, Vienna*, p. 53 (1958).
11. T. P. HILDITCH, *The Chemical Constitution of Natural Fats*, p. 465, Chapman and Hall Ltd., London (1947).
12. E. D. WILLS, *Biochem. J.* **57**, 109 (1954).
13. E. D. WILLS, *Biochem. J.* **63**, 514 (1956).
14. E. D. WILLS, *Biochem. J.* **69**, 17P (1958).
15. E. D. WILLS, *Biochem. Pharmacol.* **2**, 276 (1959).
16. F. W. PUTNAM, *Adv. Protein Chem.* IV, 79 (1948).
17. R. T. HOLMAN, *Progr. Chem. Fats & Other Lipids* **2**, 51 (1954).
18. C. D. WAGNER, H. L. CLEVER and E. D. PETERS, *Analyt. Chem.* **19**, 980 (1947).
19. K. M. WILBUR, F. BERNHEIM and O. W. SHAPIRO, *Arch. Biochem.* **24**, 305 (1949).
20. E. H. FARMER and D. A. SUTTON, *J. Chem. Soc.* p. 119 (1943).
21. R. L. PECK, *J. Amer. Chem. Soc.* **64**, 487 (1942).
22. A. L. TAPPEL, *J. Biol. Chem.* **217**, 721 (1955).
23. M. E. CLUBB and E. D. WILLS, *Biochem. J.* **71**, 16P (1959).

Biochemical Pharmacology, 1961, Vol. 7, pp. 17–22. Pergamon Press Ltd., Printed in Great Britain.

THE EFFECT OF CHLORTHIAZIDE ON HYPOXANTHINE DEHYDROGENASE, LACTIC DEHYDROGENASE, AND SOLUBLE PROTEIN OF CHICK EMBRYO KIDNEY

S. CHAUBE and C. E. CARTER

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut

(*Received* 19 *October* 1960)

Abstract—Chlorthiazide was administered to chick embryos *in vivo* and hypoxanthine dehydrogenase activity, lactic dehydrogenase activity, and the electrophoretic characteristics of the soluble fraction of embryonic renal tissues were subsequently examined. The soluble protein fraction of embryonic renal tissue was altered in embryos which had been treated with chlorthiazide, and this change was associated with a large decrease of hypoxanthine dehydrogenase specific activity.

INHIBITION of avian kidney hypoxanthine dehydrogenase by chlorthiazide *in vitro*[1] indicated that the affinity of the drug for renal enzymic protein might be sufficiently high to produce, *in vivo*, effects upon the developing embryonic chick kidney. In this paper the results of experiments are reported in which chlorthiazide was injected into the chick embryo and the activities of hypoxanthine dehydrogenase and lactic dehydrogenase, and the electrophoretic characteristics of the embryonic renal soluble proteins, were determined. It was found that the renal embryonic tissue was sensitive to the actions of chlorthiazide with a resultant depression of hypoxanthine dehydrogenase activity and an associated change in the electrophoretic pattern of the soluble tissue protein. Under certain conditions, lactic dehydrogenase activity was moderately depressed by chlorthiazide, but changes in the specific activity of this enzyme were relatively small as compared to that observed with hypoxanthine dehydrogenase.

METHODS

Materials

Chlorthiazide was purchased from Merck, Sharp and Dohme Company, diphosphopyridine nucleotide (DPN) and the reduced coenzyme (DPNH) from Pabst Laboratories.

Rhode Island Red eggs were obtained from a local farm and were incubated at 37·8 °C in a constant temperature wooden incubator with humidity adjusted at approximately 60 per cent. To insure optimal growth the eggs were rotated once daily.

All instruments used were placed in 90 per cent alcohol for 1 hr before use.

Embryos

Embryos were classified according to the stages proposed by Hamburger and Hamilton[2] for the normal development of chick. The average incubation time quoted by these authors for each stage was used; thus, the morphological development is expressed as corrected incubation time.

Injection of eggs

After the desired period of incubation, the eggs were candled to reveal the eyeball of the embryo reflected on the shell as a prominent black spot and the allantois as a faintly outlined circular vesicle. The restricted extra-embryonic area between these two structures was used for making the injections into the egg. Through a small aperture in the shell in this area, 0·1 ml of chlorthiazide solution containing the appropriate concentration of the drug in water, was slowly injected into the egg. After injection, the aperture in the shell was swabbed with alcohol, sealed with a piece of scotch tape, and the eggs were transferred to the incubator. During this operation damage to the embryo or yolk sac was carefully avoided and embryos which were accidentally injured were discarded. Control embryos were injected with 0·1 ml of distilled water alone.

Preparation of extracts of embryonic renal tissue

Embryos and membranes were removed from the egg and washed with ice-cold Ringer solution. The embryos were then separated from accessory membranes, weighed and transferred to a dissecting dish. The kidneys, including metanephric and mesonephric tissues, were removed from the surrounding structures and weighed. Approximately 0·5 g of tissue was extracted in a glass homogenizer in 1·5 ml of a 0·05 M solution of sodium phosphate buffer (pH 8·0) at 0 °C and the homogenate was centrifuged for 20 min at 34,000 **g** at 4° C. The supernatant solution was pipetted into 1-ml glass tubes immersed in an ice bath. Enzyme assays were performed on fresh supernatant fractions of homogenates. Embryonic liver extracts were prepared by the same procedure.

Hypoxanthine dehydrogenase

The enzyme activity, which occurred only in the kidney supernatant fraction, was measured by the increase in absorption at 340 mμ resulting from the obligatory reduction of DPN[1] which occurs during the oxidation of hypoxathine. The reaction mixture contained 0·05 μmoles of DPN, 0·05 μmoles of hypoxanthine, 1·0 ml of 0·1 M solution of tris buffer (pH 8·0) and an aliquot of the tissue extract (0·1 or 0·2 ml). Incubations were carried out in 1-ml silica cuvettes at room temperature in the ultraviolet spectrophotometer and absorbancy was determined at 340 mμ, in comparison with a blank which contained the above mixture except for the enzyme solution. The absorbancy of each sample was read at zero time, at 5 min, and then at regular intervals of 10 min, for a total period of 40 min.

Lactic dehydrogenase

Lactic dehydrogenase activity, which was found only in the supernatant fraction, was measured by the decrease in absorption at 340 mμ which results from the enzymic oxidation of the reduced coenzyme, DPNH.[1] The reaction system was composed of 0·5 μmoles of sodium pyruvate, 0·5 μmoles DPNH, 1·0 ml of 0·05 M tris buffer (pH 7·8) and 0·1 ml of the enzyme solution; the final volume was 1·2 ml. The absorbancy of the reaction mixture was compared with that of a blank containing all of the above components except DPNH. Since the activity of this enzyme is higher than that of hypoxanthine dehydrogenase, the supernatant fraction of the renal tissue was diluted (usually 1:80) in order to obtain rates of DPNH disappearance; these were

linear for a 10-min observation period. Activity of the enzyme was then expressed as μmoles of DPNH-decrease/min per mg of soluble protein.

Protein determination

The Folin–Ciocalteau method for determining proteins, as modified by Sutherland *et al.*,[3] was employed.

Starch block electrophoresis was carried out on extracts of embryonic kidney by the method described by Paigen,[4] and Bloemendal.[5]

RESULTS

Fig. 1 depicts the effects of several concentrations of chlorthiazide, injected into the embryo on the fifth day of incubation, upon hypoxanthine dehydrogenase activity of

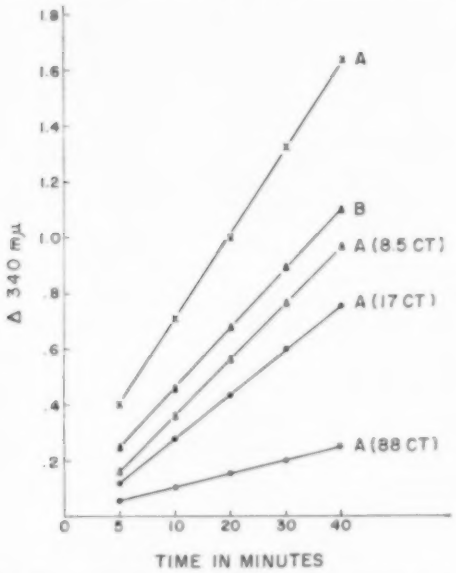


FIG. 1. Hypoxanthine dehydrogenase activity, estimated as increment of optical density at 340 mμ (DPNH) over a 40-min incubation period with supernatant fractions of embryonic kidney. Embryos were injected with three dosages (88, 17 and 8·5 μmoles) of chlorthiazide (CT) per egg on the fifth and harvested on the eighth day of incubation. Kidney supernatant fraction aliquots (A, 0·2 ml; B, 0·1 ml), prepared from 0·3 g of tissue from untreated 8-day embryos, were compared with 0·2-ml aliquots of the supernatant fraction prepared from an equivalent weight of embryonic renal tissue of chlorthiazide-treated eggs (A, CT, 8·5; A, CT, 17; and A, CT, 88).

kidneys removed on the eighth day of incubation. These data show that the formation of DPNH in the enzymatic reaction increased linearly over the 40-min period of the assay, and that enzyme activity was proportional to the amount of the supernatant fraction added. In these experiments, chlorthiazide produced over 50 per cent inhibition of renal hypoxanthine dehydrogenase activity when 17 μmoles of the compound were injected per egg.

In the chicken embryo, renal excretory function is carried out principally by the mesonephric kidney until the twelfth to fifteenth day when the metanephric kidney becomes functional.[6] The two renal organs are separable and since both function in

the 15-day embryo, the effect of chlorthiazide on hypoxanthine dehydrogenase in these tissues was examined. A comparison of the activities of hypoxanthine dehydrogenase and lactic dehydrogenase was also made, as shown in Table 1. Preliminary experiments indicated that in the 15-day-old egg 43 μmoles of chlorthiazide produced from 60 to 70 per cent inhibition of hypoxanthine dehydrogenase and this was compatible with the survival of over 90 per cent of the embryos; these conditions also

TABLE 1. THE EFFECT OF CHLORTHIAZIDE ON HYPOXANTHINE DEHYDROGENASE AND LACTIC DEHYDROGENASE OF RENAL TISSUE FROM 15-DAY EMBRYOS*

	Hypoxanthine dehydrogenase specific activity		Lactic dehydrogenase specific activity	
	Mesonephros	Metanephros	Mesonephros	Metanephros
Control†	21×10^{-3}	17×10^{-3}	2·0	1·4
CT-Day 5‡	3×10^{-3}	11×10^{-3}	1·2	1·5
CT-Day 14§	7×10^{-3}	6×10^{-3}	1·9	1·1

* Fourteen embryos were employed in each experimental group. Mesonephric and metanephric tissues were dissected from the embryo and 0·3 g mesonephric tissue and 0·9 g metanephric from each group were separately pooled and extracted with 1·0 ml of 0·1 M Tris buffer, pH. 7·8. The preparation of the supernatant fraction and definition of specific activity are described in the Methods section.

† Separate groups of control embryos were injected with 0·1 ml of distilled water on day 5 and on day 14 and both groups were harvested on day 15. Since differences in the hypoxanthine and Lactic dehydrogenases between the two groups was in the range of $\pm$1·2–2, averages were obtained and are shown in the table.

‡ 43 μmoles of chlorthiazide in 0·1 ml of distilled water were injected on day 5 and the embryos were harvested on day 15.

§ 43 μmoles of chlorthiazide in 0·1 ml of saline were injected on day 14 and the embryos were harvested on day 15.

were achieved when 17 μmoles of chlorthiazide were injected into the 8-day egg. The data of Table 1 show that chlorthiazide administration on the fifth day of incubation produced a depression of hypoxanthine dehydrogenase activity, when this was determined on the fifteenth day in both the mesonephric and metanephric renal tissue, but that the magnitude of the inhibitory effect was greatest in the mesonephros. The activity of lactic dehydrogenase, assayed under these conditions, was decreased in the mesonephros to a lesser extent than was that of hypoxanthine dehydrogenase. Lactic dehydrogenase activity was not depressed in the metanepheric kidney. These results demonstrate that chlorthiazide can produce long-term effects upon the developing embryonic kidney, and that these effects are most pronounced in the tissue which was performing the excretory function at the time of the injection of the drug. When chlorthiazide was injected into the embryos on day 14 and the enzyme activities were assayed on day 15, lactic dehydrogenase was found to be relatively insensitive to the action of the drug. Hypoxanthine dehydrogenase activity was depressed over 60 per cent in both metanephric and mesonephric tissues under these conditions. In none of the cases of chlorthiazide-induced depression of hypoxanthine dehydrogenase activity *in vivo* was there evidence of reversibility of the inhibition upon addition of excess substrate. *In vitro*, the inhibition was found to be reversible.[1]

The specific activity of the enzymes reported in Table 1 was based upon the amount of soluble protein of the supernatant fractions of kidney tissues. From these data, it

appeared that injection of the drug might be capable of altering the soluble protein component of the developing embryonic kidney. Such an effect was demonstrated in the diagrams (Fig. 2) obtained from starch block electrophoresis[4, 5] of embryonic renal tissues under experimental conditions similar to those described in Table 1. Electrophoresis of supernatant fractions was conducted on extracts prepared from the entire embryonic kidney, which included mesonephric and metanephric tissue, because of the requirement of this technique for larger amounts of starting material. The protein diagram (Fig. 2) of the supernatant fractions, prepared from equivalent

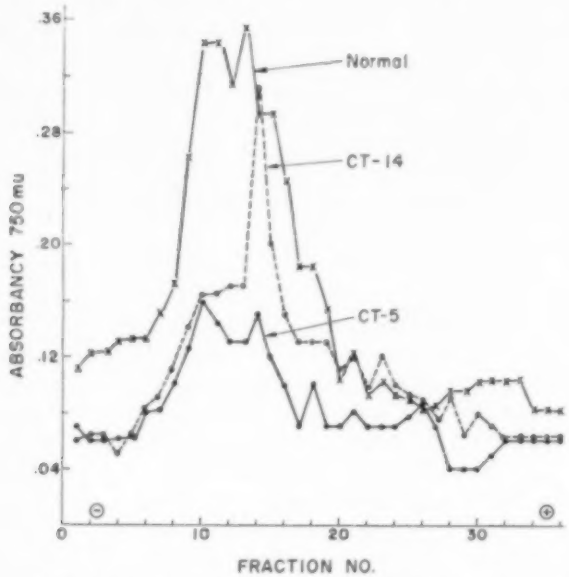


FIG. 2. Electrophoresis of the soluble proteins of the supernatant fraction of control and chlorthiazide treated 15-day embryonic kidneys. Metanephric and mesonephric tissues were combined and in each experimental series 1·2 g of tissue derived from fourteen to sixteen embryos were homogenized in 2·0 ml of 0·1 M Tris buffer, pH 7·8. The amount and time of injection of chlorthiazide were the same as described in Table 1. The starch block electrophoresis was conducted on 1 ml of the supernatant fraction for 22 hr at 400 V in the apparatus described by Paigen,[4] as modified by Bloemendal,[5] using 36-cm troughs. The buffer system employed was 0·1 M Tris, pH 7·8. From the starch blocks, 1-cm sections were cut and eluted in distilled water, 2·0 ml, 4 °C for 24 hr. Eluates were examined spectrophotometrically for absorbancy at 260 mμ (nucleic acid), and 0·2-ml aliquots of a 1 : 10 dilution of the eluate were employed for protein determination. The amount of protein in the fractions is expressed as 750 mμ-absorbancy of the colored product of the Folin reaction.[3] The diagram designated CT-14 represents the distribution of acid-soluble protein from the renal supernatant fraction of embryos which had received 43 μmoles of chlorthiazide per egg on day 14 and were harvested on day 15. The designation CT-5 represents the results of an experiment in which 43 μmoles of chlorthiazide were administered on day 5 and the embryos were harvested on day 15.

weights of control and chlorthiazide-treated embryos, demonstrated alterations attributable to the action of the drug. Embryos injected on the fifth day of incubation with 43 μmoles of chlorthiazide and harvested on the fifteenth day for preparation of renal extracts, showed an extensive and uniform depression of the principal components of the electrophoretic diagram. Embryos injected on the fourteenth day with

chlorthiazide (43 μmoles) and harvested on the fifteenth day showed a great decrease in the 9-cm component and a relatively smaller depression of the 14-cm component. Since the same weight of embryonic kidney was used in each preparation, it is apparent that the administration of chlorthiazide was associated with changes in the soluble protein components of embryonic chick renal tissue. Since both hypoxanthine dehydrogenase and lactic dehydrogenase exhibited peak specific activity in the 9-cm fraction, the electrophoretic data offer no explanation for the selective depression of the hypoxanthine enzyme (Table 1). However, the relatively low activity of hypoxanthine dehydrogenase, as compared with that of dehydrogenase in the normal tissue, may make changes in the ratio of enzyme protein to total protein, as measured by catalytic activity, disproportionately large.

The pattern of nucleic acid distribution determined by the absorption at 260 mμ of the electrophoretically separated extract, revealed a single peak with maximum absorption in the 11-cm fraction. Samples from the drug-treated tissues which exhibited the change in protein distribution shown in Fig. 2, showed no alteration in the nucleic acid distribution and no decrease in the amount of nucleic acid, as evidenced by the absorption at 260 μm.

DISCUSSION

Administration of chlorthiazide to chick embryos *in vivo* produced alterations in the proteins of the supernatant fraction of renal extracts. A decrease in hypoxanthine dehydrogenase activity was an indication of the change, but was probably a relatively non-specific consequence of the action of the drug. Under appropriate conditions a lesser effect was observed on lactic dehydrogenase, an enzyme which exhibits high activity in the renal supernatant fractions. The mechanism of the effects of chlorthiazide on the soluble proteins of embryonic chick renal tissue is not known. However, the data presented in this paper demonstrate the sensitivity of the developing chick kidney to the effects of this drug and the suitability of the tissue for investigation of the effects of chlorthiazide on the synthesis of renal soluble protein. Whether effects of a similar nature could be obtained in mammalian tissues remains to be determined, but certain observations indicate that analogous behavior would not be anticipated. First, renal hypoxanthine dehydrogenase, an enzyme obligatorily linked to DPN, does not occur in mammalian tissues.[1] Second, the observation that the extensive use of chlorthiazide in pregnancy in human subjects has not been associated with complications of fetal life, would tend to indicate that the developing chick kidney is uniquely sensitive to the actions of the drug. This sensitivity may be attributed to the metabolic characteristics of the chick kidney, or to the "closed" system of fluid cf compartments in the egg which permit attainment of high concentrations of chlorthiazide at embryonic renal sites, a circumstance not encountered in the mammalian embryo because of the placental and maternal circulation.

REFERENCES

1. E. LANDON and C. E. CARTER, *J. Biol. Chem.* **235**, 819 (1960).
2. V. HAMBURGER and H. L. HAMILTON, *J. Morph.* **88**, 49 (1951).
3. E. W. SUTHERLAND, C. F. CORI, R. HAYNES and N. S. OLSEN, *J. Biol. Chem.* **180**, 825 (1949).
4. K. PAIGEN, *Analyt. Chem.* **28**, 284 (1956).
5. H. BLOEMENDAL, *J. Chromatog.* **2**, 121 (1959).
6. A. L. ROMANOFF, *The Avian Embryo*. Macmillan, New York (1960).

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 23–30. Pergamon Press Ltd., Printed in Great Britain.

DÉTERMINATION DES ACIDES NUCLÉIQUES DE *SACCHAROMYCES ITALICUS* (CASTELLI) TRAITÉS PAR L'OXYDE DE CARBONE (CO), LE CYANURE DE POTASSIUM (CNK) OU L'AZIDE DE SODIUM (N_3Na)

A. Jeney, A. Szendrey et E. Vig

Institut d'Hygiène, l'Université de Debrecen, Hongrie

(*Received* 10 *November* 1960)

Abstract—Qualitative and quantitative determinations have been made of the nucleic acids in *Saccharomyces italicus* Castelli, subject to the action of CO, KCN, and NaN_3; and an analysis using the isotope ^{32}P has been made. According to the results:

(1) The total quantity of nucleic acids increased only under the influence of CO; under that of KCN it remained constant; while under that of NaN_3 it decreased.

(2) The quantity of DNA increased 18–19 times under the influence of CO, and 10 times under that of KCN; NaN_3 had only a slight effect.

(3) The quantity of phosphoprotides (PP) diminished by about 65 per cent under the influence of NaN_3. It remained constant under the influence of the other materials.

(4) The quantity of RNA increased by 33 per cent, compared with the controls, under the influence of CO; it was unchanged by KCN; and decreased by 60 per cent under the influence of NaN_3.

(5) The amount of phosphorus intermediates (IMP) was greatest in the controls: the decrease was about 35 per cent for cells treated with CO, about 34 per cent for KCN, and about 73 per cent for NaN_3.

(6) Phosphorus incorporated in the culture medium decreased on the average from 7 mg per g dry weight for controls to 14·41 mg per g after treatment with CO, and to 5·40 after NaN_3.

(7) The results obtained by isotopic analysis agreed with these figures, except for the value of the IMP, which increased moderately (by 10 per cent). It is interesting to note that the DNA increase was more marked in the presence of CO (63 per cent) than in that of KCN.

Dans nos recherches précédentes, nous avons examiné le comportement de *Saccharomyces cerevisiae* et de *Saccharomyces italicus* Castelli sous l'action de CO, de CNK et de N_3Na à l'aide de tubes d'Einhorn, du manomètre de Van Itterson–Kluyver, de l'appareil de Warburg, ainsi que de microcalorimètre.[1–4] Nous avons également étudié l'action bactério- et fungistatique de CNK et de N_3Na.[5]

MATÉRIAUX ET MÉTHODES

Nous avons utilisé pour nos recherches des cellules séchées de *Saccharomyces italicus* Castelli, cultivées sur gélose au marc (de malt) puis récoltées, lavées plusieurs fois et enfin centrifugées. Dans des Erlenmeyers stériles de 50 ml, nous avons introduit 20–20 ml de milieu de culture au pH 6, contenant 10% de glucose, puis nous les avons

ensemencés par 0,1 g de cellules sèches de levures, âgées de 2 semaines au plus. Les concentrations des réactifs ajoutés au milieu de culture étaient de: mole 1000 pour le CNK et le N_3Na, de 2% pour le gaz CO, donc les mêmes que dans nos expériences précédentes.[1-3] Nous avons produit le gaz CO par la réaction de l'acide sulfurique sur l'acid formique, tout deux étant analytiquement purs.

Le gaz ainsi obtenu a été lavé successivement dans une solution de soude caustique, puis dans l'eau distillée pour enlever les impuretés acidiques éventuelles. Le gaz ainsi purifié a été ensuite introduit dans de l'eau distillée stérile de façon à obtenir une solution aqueuse saturée c'est à dire le 2% environ. La concentration de CO a été déterminée par la méthode de Wennesland.[6] Dans les récipients de contrôle nous n'avons mis que du milieu de culture stérile et 0,1 g de cellules sèches de levures. L'incubation a été de 48 heures à 26 °C. Après ce délai, le contenu des récipients est centrifugé et séché, puis le poids du sédiment sec est mesuré. Nous avons examiné la teneur en acides nucléiques d'environ 440–480 millions de celleules de levure (dans 1 ml) traitées par différents réactifs. La détermination des proportions des différentes réactifs. La détermination des proportions des différentes fractions nucléiques a été faite selon la méthode de Schmidt-Tannhauser (S.T.), combinée à celle de Schneider[7] (Sch.).

La combinaison des deux méthodes permet d'éliminer plusieurs inconvénients propres à chacune d'elles isolement.[8] Avec la méthode "Sch.", l'ADN n'influence pas la réaction à l'orcine de l'ARN. D'autre part, les valeurs des phospho-protéines ne sont pas aussi anormalement élevées que dans le cas de la seule méthode "Sch.". Tandis que la méthode "S.T." est basée sur la détermination du phosphore des différentes fractions, la méthode "Sch." distingue le ribose et le désoxyribose par leurs réactions colorées. Les nucléotides sont déterminés par leurs pentoses et non pas par contenu en phosphore.

Vol. 7
1961

SCHÉMA DE LA MÉTHODE "S.T."

Le résidu sec, en poudre, des cellules de levure, séchées et mesurées, est dissous dans la soude caustique et mis en incubation pendant un certain temps.

Solution "a"

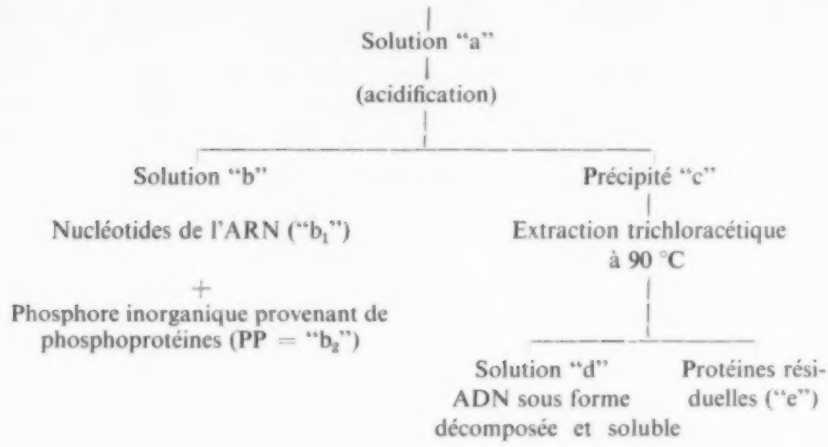
(acidification)

Solution "b"	Précipité "c"
Nucléotides de l'ARN ("b_1")	Albumines + ADN
+	("e") ("d")
Phosphore inorganique provenant des phosphoprotéines (PP = "b_2")	

Détermination du phosphore par la méthode de Robinson-Marland.

SCHÉMA DE LA MÉTHODE "S.T." ET "SCH." COMBINÉE

Le résidu sec, en poudre, des cellules de levure, séchées et mesurées, est dissous dans la soude caustique.



Nous avons répeté nos examens à plusieurs reprises. Nous donnons nos résultats en valeurs moyennes. Ces expériences ont été complétées par des examens exécutés à l'aide de l'isotope ^{32}P. Les cellules de levure ont été cultivées sur un gélose au marc de malt, contenant 5 μc de ^{32}P pour 5 ml de milieu. Les fractions nucléiques des cellules de levure étudiés sont les suivants: (a) tout les acides nucléiques (AN) = ARN + ADN (= "b_1" + "d"); (b) ARN + P inorg.; (c) = ADN ("d")+ protéines residuelles ("e"); (f) phosphore intermédiaire (IMP) = phosphore ne provenant pas d'acides nucléiques (P total = "a"). Phosphoprotéines (PP) = le contenu en phosphore des protéines ("b_2").

RÉSULTATS

1. Dans nos expériences, exécutées par la *première méthode*, nous avons trouvé, que la quantité totale des acides nucléiques n'augmentait que sous l'action de CO (51%). Elle est restée presque inchangeé sous l'action de CNK, tandis que, sous l'action de N_3Na, nous avons constaté une diminution nette (65%).

La quantité de l'ADN n'a changé pratiquement que très peu sous l'action de N_3Na et encore elle a plutôt diminuée. Dans les séries traitées par le CNK, la quantité de l'ADN a été presque 10 fois plus grande et, sous l'influence de CO, elle est même devenue 18–19 fois plus grande. Dans les valeurs des phosphoprotéides, nous n'avons trouvé de changement qu'en cas de traitement au N_3Na: il s'agissait d'une diminution de 65%.

La quantité de l'ARN a diminué de 60% par rapport aux contrôles sous l'action de N_3Na. Elle est restée presqu'invarieé sous l'action de CNK, et montrait une augmentation de 33% sous l'influence de CO. La quantité du phosphore intermédiaire la plus élevée a été trouvée dans les contrôles. Nous avons trouvé une diminution de 34% dans les cas des cellules traitées par CNK, de 35% pour celles traitées par N_3Na. La diminution du phosphore incorporé au milieu de culture est montée jusqu à 7 mg/g de substance sèche dans les contrôles, jusqu' a 5,40 mg/g après un traitement de N_3Na et 10,41 mg/g après celui de CO. L'action de CNK n'a pu être évaluée de ce point de vue (voir Tableaux 1 et Fig. 1).

2. Nous avons completé nos expériences par des examens exécutés à l'aide de l'isotope ^{32}P. Nous avons incorporé 5 μc de ^{32}P au milieu au marc de malt gélosé réparti dans des tubes inclinés. Après l'incubation, nous avons enlevé les colonies avec précaution à l'aide d'une anse. Il faut éviter de toucher la gélose elle-même. Nous avons lavé les cellules à plusieurs reprises ou moyen de soluté physiologique,

TABLEAU 1.

	Contrôles	N_3Na (mg/g de substance seche)	CNK	CO
Nombre des cellules	480×10^6	450×10^6	500×10^6	486×10^6
Quantité totale des acides nucléiques	7,53	2,60	8,07	11,38
ADN	0,11	0,09	1,03	2,11
PP	1,07	0,30	1,25	1,29
ARN	6,91	2,37	6,91	9,21
P intermédiaire	4,91	1,15	3,41	3,05
Teneur en P du milieu au 0 heure	43,50	43,50	43,50	43,50
Teneur en P du milieu au 48 heures	36,42	38,10	Non évaluable	33,09
Diminution du P du milieu	7,08	5,40	Non évaluable	10,41

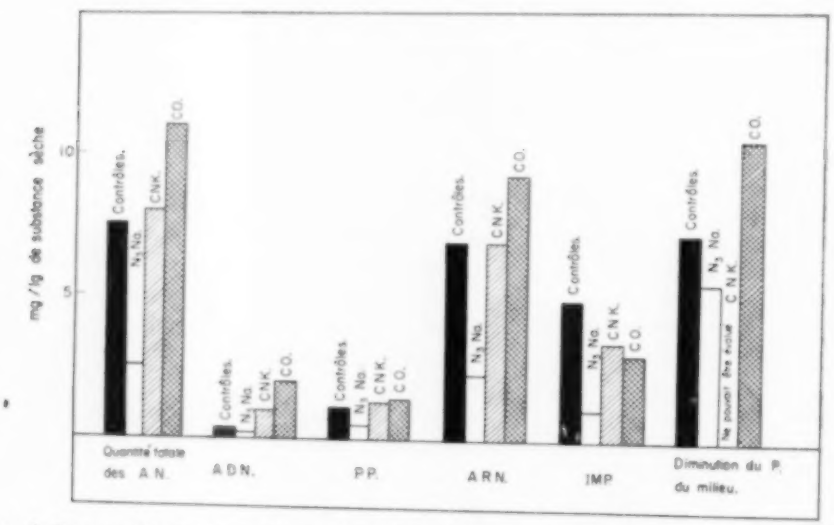


FIG. 1. Examens des acides nucléiques des cellules de levure. Concentrations: N_3Na, mol/1000; CNK, mole/1000; CO, 2%.

jusqu'au moment où les eaux mères ne montraient plus d'activité radioactive. En mesurant l'activité propre des cellules même, nous avons trouvé qu'elle correspondait à une activité de 2 μc. Les traitements par les divers réactifs ont été les mêmes que dans les experiences précédentes. Après avoir établi le nombre des cellules et leur résidu sec, nous avons séparé les acides nucléiques par la méthode combinée Schmidt–Tannhauser–Schneider, puis nous avons mesuré l'activité des différentes fractions.

Vol. 7
1961

RÉSULTATS

Nos résultats sont représentés dans le Tableau 2 et la Fig. 2. (Ce sont des valeurs moyennes de quatre examens parallèles, exécutés en deux séries d'expériences.)

Les changements produits dans les différentes fractions sous l'influence des traitements de N_3Na, de CNK et de CO figurent au Tableau 2, où ils sont indiqués par rapport au contrôle pris chaque foi comme unité.

TABLEAU 2

	Contrôles	N_3Na	CNK	CO
Quantité totale des acides nucléiques	1,000	− 0,660	+ 0,151	+ 0,315
ADN	1,000	− 0,523	+ 1,130	+ 2,280
PP	1,000	− 0,661	− 0,054	+ 0,040
ARN	1,000	− 0,669	+ 0,025	+ 0,088
IMP	1,000	+ 0,109	− 0,092	− 0,098

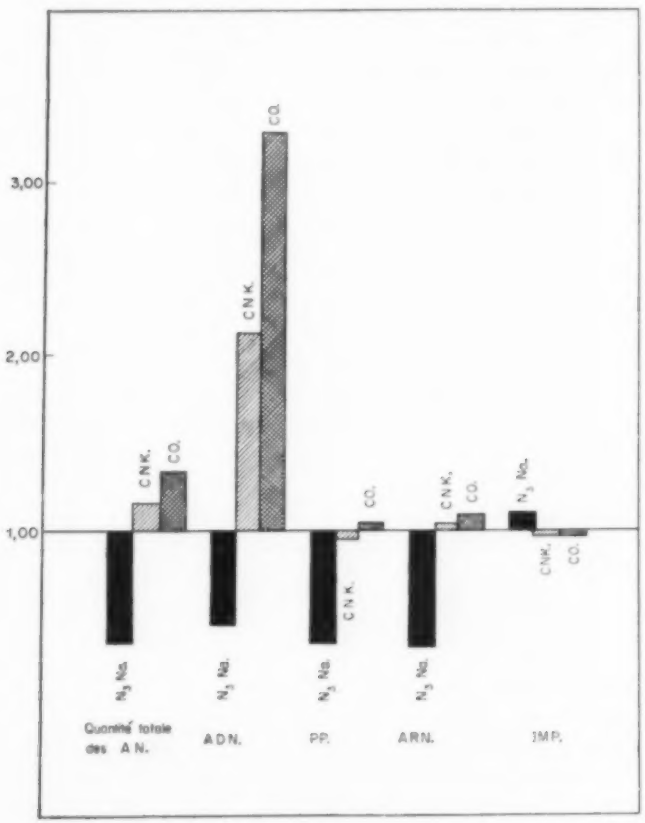


FIG. 2. Examens de *Saccharomyces cerevisione* a l'acide de l'isotope ^{32}P. changement des résultats par rapport aux contrôles.

Tout en admettant, que la séparation des différentes fractions des acides nucléiques peut ne pas être parfaitement exacte, il apparaît, néanmoins, que la quantité totale des acides nucléiques a augmenté sous l'effet de CNK de 15% et sous celui de CO de 32%, tandis qu'elle a diminué de 66% environ sous l'effet de l'azide. De même, sous l'action de N_3Na, il y a une diminution de l'ADN, de PP et de l'ARN (de 52, de 66 et de 67%, respectivement). La valeur de l'IMP est cependant augmenté de 11%. Sous l'action de CNK, la quantité de l'ADN augmente de 113%, tandis que celle l'ARN n'est augmentée que de 2,5%. Les quantités de PP et de l'IMP diminuent de 5,4 et 9%, respectivement. Sous l'action de CO la quantité d'ADN augmente de 228%, celle de PP, de 4% et l'ARN de 9%, tandis que l'IMP diminue de 9,8%. Par conséquent, nous pouvons conclure que l'action de CO est à peu près égale à celle de CNK, tandis que N_3Na exerce une action toute opposée. Il est remarquable, que, sous l'effet du traitement par CO, l'augmentation de l'ADN soit de 63% plus grande que sous l'action de CNK.

DISCUSSION DES RÉSULTATS

Au cours de nos propres experiences nous avons remarqué une différence nette entre les actions de N_3Na, CNK et surtout CO, sur le métabolisme nucléinique des cellules de levure. N_3Na provoque une diminution de la quantité totale des acides nucléiques, de l'ADN, de l'ARN et du phosphore protéinique. Seule la valeur du phosphore intermédiaire montre une légère augmentation. Le contraste entre l'action de N_3Na et celle de CNK a été déjà traité dans un article précédent. CNK n'inhibe pas la réduction des nitrates et ne possède guère d'effet bacteriostatique ou fungistatique, contrairement à N_3Na. CNK empêche la réduction du fer ferrique, tandis que N_3Na inhibe l'oxydation du fer ferreux. D'après nos expériences, nous pouvons ajouter à ces différences celle qui se traduisent nettement par l'influence exercée sur le métabolisme nucléinique. Du fait que l'action de CNK est semblable à celle de CO, on pourrait supposer que les deux substances réagissent de la même façon, c'est à dire qu'elles empêchent la respiration, l'oxydation directe du glucose, ainsi que la formation d'acide gluconique en présence d'oxygéne. On pourrait chercher la cause des différences existant entre l'action de ces deux substances, d'une part, et celle de N_3Na, d'autre part, dans le fait que ce dernier inhibe la synthèse des hydrates de carbone mais n'inhibe pas la fermentation anaérobique.

Étant donné, cependant, que l'action de CO est beaucoup plus évidente que celle de CNK, il faut chercher d'autres explications aussi. Selon nos connaissances actuelles, CO_2 est utilisé par tout organisme pour la formation du noyau pyrimidique par l'intermédiaire de phosphate de carbamyle ($NH_2COOP_3H_2$). La réduction de CO_2 ou des bicarbonates par la déhydrogénase formique ubiquitaire conduit à la formation des formiates. C'est le DPNH qui joue ici le rôle de donneur de H_2.

Pour nos recherches futures, nous avons adopté l'hypothèse suivante: la formation des formiates peut se faire aussi bien à partir de CO qu'à partir de CO_2. Pour la formation du soi-disant "formiate actife", c'est l'acide tétrahydrofolique qui est responsable (THFA).[9] A côté de "l'acétate actife" (acétyl CoA), c'est cette substance qui joue un rôle primordial au cours des biosynthèses. Il est connu, que le "formiate actife" occupe une place centrale au cours du métabolisme de l'acide formique, de la sérine, du glycocolle, de l'acétone, de la méthionine (créatinine) et des nucléotides.

Vol. 7
1961

Les publications récentes de Goldwait[10] de Levenberg et Buchanan[11, 12] prouvent aussi le rôle de la formylation dans la formation des noyaux puriques.

Nos propres expériences prouvent aussi que la biosynthèse des acides nucléiques peut être facilitée non seulement par la présence du formaldéhyde ou des formiates ou, éventuellement, de "formiate actife" provenant de ceux-ci, mais aussi par CO, qui peut être également la source de la formation de formiates.

Sous l'influence de CO, c'est surtout la production d'ADN qui a augmenté. L'augmentation correspondant obtenu par l'action de CNK est deux fois moindre.

Nous avons constaté, au cours de nos expériences précédentes, que CO favorise la fermentation du glucose. Son activité sur le métabolisme nucléinique est encore plus marquée. Ceci est d'autant plus important que nous ne connaissons pas d'autres substances qui provoquent un aussi grand changement dans la répartition des acides nucléiques et qui entrainent, notamment, une telle augmentation de l'ADN.

Nos constatations ouvrent dès lors une nouvelle voie et donnent un aspect nouveau non seulement au métabolisme des cellules de levure, mais aussi à celui d'autres micro-organismes, et même à celui des cellules végétales et animales.

Nos recherches portant sur le même sujet, mais poursuivies avec un matériel très diffirent, des tissus tumoraux provenant de tumeur d'ascites d'Ehrlich son actuellement en cours.

ol. 7
961

SOMMAIRE

Les auteurs ont déterminé, par des essais quantitatifs et qualitatifs, les acides nucléiques de *Saccharomyces italicus* Castelli soumis à l'action de CO, CNK et N_3Na. Il sont completé leurs examens par des essais exécutés à l'aide de l'isotope ^{32}P. Selon leurs résultats:

(1) La quantité totale des acides nucléiques n'a augmenté que sous l'influence de CO. Sous l'influence de CNK elle est restée constante, tandis que sous celle de N_3Na elle a diminué.

(2) La quantité d'acide désoxyribonucléique (ADN) a augmenté de 18 a 19 fois sous l'influence de CO et de 10 fois sous celle de CNK. Pratiquement, N_3Na n'a influencé que très peu la quantité d'ADN.

(3) La quantité des phosphoprotéides (PP) a diminué de 65% environ sous l'influence de N_3Na. Elle est restée à peu près constante sous l'influence des deux autres produits.

(4) La quantité d'acide ribonucléique (ARN) a augmenté (par rapport aux contrôles) de 33% sous l'influence de CO, elle n'a pas changé sous celle de CNK et elle a diminué de 60% sous celle de N_3Na.

(5) Le taux de phosphore intérmédiaire (IMP) était le plus élevé chez les contrôles. La diminution était d'environ 35% pour les cellulés traitées au CO, de 34% pour celles traitées par CNK et de 73% pour celles traitées au N_3Na.

(6) Le phosphore incorporé au milieu de culture a diminué, en moyenne, de 7 mg/g de substance sèche dans les cas des contrôles, de 10,41 mg/g aprés traitement au CO et de 5,40 mg/g après traitement au N_3Na.

(7) Les résultats moyens des essais exécutés avec du phosphore isotope ^{32}P correspondaient aux précédents, à l'exception de la valeur du phosphore intermédiaire, qui a montré dans cette série d'expériences une augmentation modérée (11%). Il

convient de remarquer que l'augmentation de l'ADN a été plus accentuée sous l'action de CO (63%) que sous celle de CNK.

BIBLIOGRAPHIE

1. E. JENEY, S. SZENDREY et É. DÁVID, *Acta Physiologica Acad. Sci. Hung.* Suppl. **5**, 51 (1954).
2. E. JENEY et S. SZENDREY, *Gigienia i Szanitarija* No. 11, 36 (1956).
3. E. JENEY et S. SZENDREY, *Egészségtudomány* **3**, 59 (1959).
4. E. JENEY, B. PAZONYI et S. SZENDREY, *Zbl. Bakt.* II Orig. (1960). Sous presse.
5. E. JENEY et T. ZSOLNAI, *Zbl. Bakt.* I Orig. **171**, 117 (1957).
6. R. WENNESLAND, *Acta Phys. Scand.* **1** (1940).
7. W. C. SCHNEIDER, *J. Biol. Chem*, **161**, 293 (1945).
8. B. TANKÓ, *Magyar Kémikusok Lapja* **12**, 277 (1957).
9. G. K. GREENBERG, *J. Biol. Chem.* **190**, 611 (1951).
10. D. A. GOLDTWAIT, *J. Biol. Chem.* **221**, 555, 569, 1071 (1956); *Ibid.* **222**, 1051 (1956).
11. B. LEVENBERG et J. M. BUCHANAN, *J. Amer. Chem. Soc.* **78**, 504 (1956).
12. B. LEVENBERG et J. M. BUCHANAN, *J. Biol. Chem.* **224**, 1015, 1019 (1957).

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 31–34. Pergamon Press Ltd., Printed in Great Britain.

THE OXIDATION OF N-*p*-BENZOTRIDEUTERIDESULFONYL N′-*n*-BUTYLUREA (TOLBUTAMIDE-D_3) BY MAN

R. U. LEMIEUX*, K. F. SPOREK*, I. O'REILLY† AND E. NELSON†

* Department of Chemistry, University of Ottawa, Ottawa, Ontario and † School of Pharmacy, University of California Medical Center, San Francisco 22, California

(*Received* 27 *October* 1960)

Abstract—The rate of the *in vivo* oxidation of the antidiabetic drug tolbutamide (N-*p*-toluenesulfonyl-N′-*n*-butylurea) to N-*p*-carboxybenzenesulfonyl-N′-*n*-butylurea, as measured by the rate of the excretion of the oxidation product, was essentially unaffected by complete deuteration of the site of oxidation.

THE mechanism of the disposition of this compound involves oxidation of the methyl group of the toluene residue to a carboxyl group. Therefore it was reasonable to expect that, if the breaking of a C—H bond of the methyl group is involved in the rate-controlling step of the biological oxidation, replacement of the hydrogens of the methyl group of deuterium would decrease substantially the rate of the oxidation. In fact, the difference in reactivity could be in the order of from 7 to 9.[1] Also, it could be expected that the deuteration would not affect appreciably the antidiabetic activity of the compound.

When tolbutamide is administered to either normal or diabetic human subjects, the product of the oxidation, N-*p*-carboxybenzenesulfonyl-N′-*n*-butylurea (carboxytolbutamide), may be recovered from the urine in substantial amounts.[2] The oxidation proceeds at a moderate rate *in vivo*. The biological half-life of tolbutamide has been reported to be in the range of 4·7 hr[3] to 7 hr.[4]

The experimental conditions used for the preparation of the tolbutamide-D_3 are described in the experimental section. The nuclear magnetic resonance spectrum of the deuterated tolbutamide indicated that the extent of deuteration was 95 ± 1 per cent. Also, it was established that the procedures used in preparing the deuterated tolbutamide for ingestion caused no exchange of deuterium for hydrogen.

The following procedure was used for the tests *in vivo*. Five healthy adult human subjects ingested before breakfasting, 0·5-g doses of powdered sodiotolbutamide contained in hard gelatine capsules; food was not taken for 1·5 hr after drug ingestion. Urine specimens were, in most cases, collected 2, 4, 6, 8, 10, 12, 14, 24 and 36 hr after drug ingestion and assayed for carboxytolbutamide.[5] The experiment was repeated about two months later with the same test subjects taking 0·5-g doses of sodiotolbutamide-D_3 and with the collection of an additional specimen of urine after 48 hr.

For four of the five test subjects participating, the data could be treated as resulting from a first-order process. The one test subject (subject E, see Table 1) whose excretion data could not be treated in this manner gave anomalous results in both test series. In view of the established fact that carboxytolbutamide disappears from the body

approximately nine[6] to twelve[7] times faster than does tolbutamide, the rate of excretion of carboxytolbutamide can be taken as a measure of the rate of the *in vivo* oxidation of the tolbutamide. Thus, the slope, k, of the plot,

$$k\,(t_2 - t_1) = 2{\cdot}303 \log \frac{W\infty - W_1}{W\infty - W_2}$$

wherein W_1, W_2 and $W\infty$ are the amounts of carboxytolbutamide excreted after times t_1, t_2 and $t\infty$ (36 hr), respectively, can be used as a measure of the relative rates of oxidation. Since a period of 2 hr after ingestion of the dose was required for absorption of the drug and equilibrium to be established, the value for t_1 was set at 2 hr. The

TABLE 1. RATES OF EXCRETION OF CARBOXYTOLBUTAMIDE

Time (hr)	A*		B*		C*		D*		E*	
	W (mg)	k (hr^{-1})	W (mg)	k (hr^{-1})	W (mg)	k (hr^{-1})	W (mg)	k (hr^{-1})	W (mg)	k (hr^{-1})
A. Tolbutamide										
2	36		29		57		51		8	
4	112	0·116	108	0·116	147	0·139	172	0·199	41	
6	177	0·120	179	0·095	205	0·116	211	0·087	80	
8	220	0·099	239	0·120	246	0·104	239	0·070	116	
10	257	0·104	280	0·100	288	0·135	287	0·150	144	
12	291	0·120	314	0·095	308	0·075	331	0·203	171	
14	316	0·116	346	0·127	330	0·104	347	0·100	201	
24	383	0·111	424	0·119	400	0·134	375	0·050	274	
36	416		458		425		419		355	
Average k (hr^{-1})		0·112		0·110		0·115		0·123		
B. Tolbutamide-D_3										
2	35		98		65		32		26	
4	108	0·091	206	0·150	145	0·123	116	0·127	84	
6	172	0·104	273	0·123	205	0·116	186	0·139	126	
8	220	0·091	331	0·135	248	0·104	237	0·131	174	
10	256	0·083	365	0·123	284	0·108	277	0·135	201	
12	287	0·087	396	0·116	314	0·115	306	0·127	232	
14	313	0·087	421	0·120	339	0·120	325	0·108	260	
24	393	0·089	471	0·076	404	0·122	376	0·102	336	
36	431		498		420		402		370	
48	449		514		431		405		407	
Average k (hr^{-1})		0·090		0·120		0·115		0·124		

* Test subjects.

increasing amount of carboxytolbutamide, W, found in the urine at the times indicated for the four normal patients (A, B, C and D), together with the calculated velocity constants, are presented in Table 1. The half-lives for the *in vivo* oxidations of both the tolbutamide and the tolbutamide-D_3 for the four test subjects were calculated from the data in Table 1. The mean value of these half-lives are 6·0 and 6·2 hr for the oxidation of tolbutamide and the tolbutamide-D_3, respectively. Thus, these values indicate an apparent isotope effect of 1·03. Although apparently normal biolgocal variations are shown in both cases, it is obvious from these results that no significant difference exists in the *in vivo* oxidation rates of the two forms of tolbutamide. The mean half-life obtained in studies to be reported[7] from one of these laboratories (University of California Medical Center) using the same test subjects as in the work reported now but giving tolbutamide as a free acid was 5·2 hr. Using this value for the

Vol. 7
1961

mean half-life for oxidation of tolbutamide gives an apparent isotope effect of 1·19. Assuming that tolbutamide-D_3 undergoes the oxidation at the same rate as does tolbutamide and that the total amount of hydrogen in the methyl group which is attached to the benzene ring is present in this form, then the maximum apparent isotope effect may be some 15 per cent greater.

Any attempt to attach theoretical significance to the present results would necessarily be highly speculative, even if the rates determined were known definitely to be directly related to the rate-controlling stage of the biological oxidation. Presumably, this stage would be concerned with the oxidation of the methyl group in either a one- or two-electron process. Nevertheless, the results are not without interest since they do limit the possible mechanisms and can serve as a guide in future investigations related to the oxidation of tolbutamide *in vivo*. It is of interest to contrast the present results to those of Belleau *et al.*[8] They found that deuteration of the carbon *alpha* to the amine in both tyramine and tryptamine caused a two- to three-fold increase in the intensity of adrenergic responses in cats. In *in vitro* experiments, the deuteration decreased the rate of oxidation by a monoamineoxidase from rat liver by a factor of 2·3.

EXPERIMENTAL

p-*Benzotrideuteride sulfonamide*

The directions for the preparation of benzotrideuteride are an adaption of the method reported by Renaud and Leitch.[9] A mixture of acetic anhydride (freshly distilled over magnesium metal) (135 g) and 99·7 per cent deuterium oxide (30 g) was heated at a 100 °C for 1 hr. Benzotrichloride (169 g) in dry ether (200 ml) was added dropwise over a period of about 5 hr to a stirred mixture of the acetic acid-D (183 g), Merck 94 per cent pure zinc dust (183 g), and dry ether (200 ml) kept in an ice–water bath. The reaction temperature was maintained between 3 °C and 5 °C throughout the addition. After the addition was complete, the mixture was stirred at room temperature for 2 hr. Water (200 ml) was added and the ethanol layer was washed twice with water, then with a 1 per cent solution of sodium carbonate, and again with water. After drying over anhydrous sodium sulfate, the ether was removed using an efficient column for fractional distillation. The yield of benzotrideuteride deuterated with the methyl group to the extent of 95 ± 2 per cent was 60 per cent.

Benzotrideuteride (57·5 g) was heated to boiling and chlorosulfonic acid (80 ml) was added at a rate sufficient to maintain refluxing. After addition was completed the mixture was stirred for 15 min, cooled to 0 °C, and poured into 500 ml of ice–water mixture. The crystalline product was gathered and pressed as free as possible of the oily phase. After drying, the product was recrystallized from petroleum ether, after decolorization of the solution with silica gel. The total yield (61·5 g) of *p*-benzotrideuteridesulfonyl chloride, melting at 63–66 °C, was added, in lots of from 2 to 3 g, to a nearly boiling mixture of concentrated ammonium hydroxide (250 ml) and water (250 ml). After the addition was complete, the mixture was cooled to about 5 °C. The near quantitative yield of crystalline product, *p*-benzotrideuteridesulfonamide, was collected, washed with water and dried *in vacuo* at room temperature.

Tolbutamide-D_3

The above-described precursor, after conversion to the sodium salt by neutralization with sodium methoxide, was condensed with an equimolar amount of

n-butylisocyanate in acetone at from 15 to 20 °C. The tolbutamide-D_3 crystallized on neutralization with acetic acid. After washing with water, the product was dried before recrystallization from ethyl acetate–petroleum ether. *Anal.* Calcd. for $C_{12}H_{15}D_3N_2O_3S$, 11·7; N, 10·25; equiv. wt. 273·4. Found: S, 11·63; N, 10·21; equiv. wt. 274. Analysis of the compound by nuclear magnetic resonance spectroscopy indicated that 16 ± 2 per cent of the atoms substituted on the carbon of the methyl group were hydrogen. Therefore, the extent of deuteration was 95 ± 2 per cent.

Acknowledgement—The authors wish to thank the Upjohn Company for financial assistance and analytical results on extent of deuteration.

REFERENCES

1. K. B. WIBERG, *Chem. Rev.* **55**, 713 (1955).
2. L. H. LOUIS, S. S. FAJANS, J. W. CONN, W. A. STRUCK, J. B. WRIGHT and J. L. JOHNSON, *J. Amer. Chem. Soc.* **78**, 5701 (1956).
3. R. E. KNAUFF, S. S. FAJANS, E. RAMIREZ and J. W. CONN, *Metabolism* **8**, 606 (1959).
4. K. M. WEST and P. C. JOHNSON, *Metabolism* **8**, 596 (1959).
5. E. NELSON, I. O'REILLY and T. CHULSKI, *Clin. Chim. Acta* **5**, 774 (1960).
6. R. E. KNAUF, S. S. FAJANS, E. RAMIREZ and J. W. CONN, *Ann. N.Y. Acad. Sci.* **74**, 603 (1959).
7. E. NELSON and I. O'REILLY. To be published.
8. B. BELLEAU, J. BURBA, M. PINDELL and J. REIFFENSTEIN, *Science*. In press.
9. R. RENAUD and L. C. LEITCH, *Canad. J. Chem.* **34**, 98 (1956).

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 35–46. Pergamon Press Ltd., Printed in Great Britain.

RELATION OF MAST CELL CHANGES TO HYPOTHERMIA IN THE RAT

D. JAMIESON and H. A. S. VAN DEN BRENK

Radiobiological Research Unit,
Cancer Institute Board, Melbourne, Australia

(*Received* 10 *October* 1960)

Abstract—Hypothermia caused by compound 48/80 in rats appears to result from histamine and 5HT liberated from ruptured mast cells and is antagonized by a specific antihistamine, mepyramine, and by LSD without preventing the rupture of mast cells. However, hypothermia caused by CPZ and related phenothiazine derivatives which also rupture mast cells is based on a different mechanism in that: (i) the hypothermia is not antagonized by mepyramine; (ii) the hypothermia can be dissociated from histamine release by prior treatment with compound 48/80 to deplete tissue histamine and tissue mast cells; (iii) LSD abolishes the hypothermic activity of CPZ but not its mast-cell-disrupting activity. Since peripheral 5HT antagonists (BOL and UML 491) do not reduce CPZ hypothermia, the latter appears to be associated with release of central nervous system 5HT. The observation[3] that cyanide causes a reduction of both CPZ hypothermia and mast cell disruption was not confirmed.

THE mast cells are considered to be an important source of tissue histamine[1] and also 5-hydroxytryptamine (5HT).[2] Both amines, particularly the latter, administered exogenously induce hypothermia in rats, mice, and other animals. However, certain antagonists of histamine and 5HT (e.g. promethazine and chlorpromazine), also cause pronounced hypothermia. The relationship which exists between endogenous amines, mast cells and the induction of hypothermia is by no means clear. Recently Le Blanc[3] showed that chlorpromazine (CPZ) caused disruption of mast cells *in vivo* and postulated an association between the hypothermic effects of CPZ and the liberation of histamine and 5HT from mast cells. Furthermore Junqueira and Beiguelman[4] reported that cyanide prevented the mast cell disruption (*in vitro*) caused by certain histamine liberators, and Le Blanc[5] reported that cyanide reduced both the hypothermic effect and mast cell disruption *in vivo* caused by CPZ. The present paper deals with studies on the hypothermic effects of CPZ and other substances in relation to mast cell disruption, with specific reference to the effect of CPZ in histamine and 5HT depleted animals.

MATERIALS AND METHODS

Adult rats of Wistar Hooded or Canberra Black stock, 150–200 g weight were used.

Drugs and administration

All doses are expressed in terms of the corresponding salt. The following drugs were used: 2-chloro-10 (3′-dimethylamino-*n*-propyl)-phenothiazine hydrochloride (chlorpromazine CPZ); 10-(2-dimethylamino-1-propyl) phenothiazine hydrochloride

(promethazine); *dl*-10-(3-dimethylamino-2-methylpropyl) phenothiazine neutral tartrate (trimeprazine); 10-(3-dimethylaminopropyl)-2-(trifluoromethyl) phenothiazine hydrochloride (trifluprομazine); 2-trifluoromethyl-10-(3′ 1″-methyl piperazinyl-4″-propyl) phenothiazine dihydrochloride (trifluoperazine); N-*p*-methoxybenzyl-N′N′-dimethyl-N-α-pyridylethylenediamine maleate (mepyramine maleate); β-dimethylaminoethyl benzhydryl ether hydrochloride (diphenhydramine hydrochloride); D-lysergic acid diethylamide tartrate (LSD); D-2-bromolysergic acid diethylamide bitartrate (BOL); 1-methyl-D-lysergic acid-butanolamide (UML 491, UML); N-ethyl-3-piperidyl*cyclo*pentylphenyl glycolate hydrochloride (JB 329); 5-hydroxytryptamine creatinine sulphate (5HT); histamine acid phosphate; reserpine; 2 : 4 dinitrophenol (DNP); compound 48/80.

All compounds were injected intraperitoneally in either normal saline or buffered solution. The injected volume varied from 0·2–0·8 ml and control animals received an equivalent volume of saline. Where antagonism to drug action was examined, the antagonist was given 5 min prior to the injection of the agonist.

Mast cell preparations

At the end of most experiments the animals were killed by cervical fracture, and a segment of the mesentery of the terminal ileum removed without stretching by the application of two plastic rings to sandwich the sample of mesentery. The rings holding the specimen of mesentery were placed in 70 per cent ethanol and stained with 1 per cent toluidine blue. Following dehydration and clearing the samples were removed from the rings, mounted in DPX and examined microscopically for mast cell morphology. The changes in the mast cells were recorded. No attempt was made to score the morphological changes quantitatively, because as previously reported[6] such values are considered too inaccurate for critical assessment, owing to sampling difficulties. The classification of mast cells proposed by Riley[7] as Types I and II according to their vascular relationships, size and staining characteristics, has been followed in recording the changes observed after various treatments.

Temperature measurements

The rectal temperature of the rats was measured with a thermistor probe or a rectal thermometer inserted to 40 mm depth. Temperature readings were taken before, and at half hourly intervals after the administration of the test compounds. The ambient temperature was kept at 20 ± 1 °C for all experiments. In most cases groups of six rats were used for each drug treatment and the readings for each time interval were averaged and the standard error calculated. The temperature was usually recorded for 2 hr after injection of the test compound, except for reserpine where a longer time interval was used and temperature taken hourly.

Oxygen Consumption

The total oxygen consumption ($\dot{Q}O_2$) for a group of from three to six rats at a time was measured by means of a closed-circuit constant-pressure respirometer. The oxygen consumption was recorded for a 30-min run in the respirometer before and after a particular treatment. The rats were weighed before the experiment and the $\dot{Q}O_2$ expressed as the mean ml O_2 consumed/100 g rat weight per min. The reduction of $\dot{Q}O_2$ caused by treatment is expressed as a percentage reduction of control values.

Depletion of amines

For acute histamine depletion compound 48/80 was given intraperitoneally in a single dose of 200 μg. Subacute histamine depletion was carried out in accordance with the dosage schedule of Riley and West by giving 48/80 twice daily over 4 days in increasing dosage (2 $\times$ 50 μg doses first day, and increasing by 50 μg per dose per day). This schedule has been shown to reduce the histamine content of most tissues to less than 10 per cent[1] and also produces some tissue 5HT reduction.[2]

Reserpine (10 mg/kg) was given to deplete tissue 5HT. Sixteen hours after injection of reserpine rats were severely tranquillized and hypothermic and previous assays of rat tissue 5HT content have shown a reduction to 10 per cent and 30 per cent of control values for spleen and skin, respectively.[8]

Histamine content of rat mesentery

The histamine content of rat mesenteries was determined for control and treated animals. Compounds were injected intraperitoneally in doses given under results and controls were injected with equivalent volumes of normal saline solution. From 16 to 40 hr later the rats were killed by cervical fracture and the mesenteries removed, dried between filter papers and weighed. Histamine extractions and separation from impurities were carried out as described by Code and McIntire.[9] The extracts were neutralised with sodium hydroxide and diluted with Tyrode solution to the required volume. The histamine content was estimated on the atropinized guinea pig ileum suspended in Mg^{2+} free Tyrode and aerated with oxygen, using histamine acid phosphate as the standard. To ensure that the dose–response relationship was the same for both the standard and test substance several dilutions of each were tested after matched responses were obtained. Extracts were retested after inhibition of histamine by mepyramine (5×10^{-8} g/l.) and no contraction resulted.

RESULTS

Effects of compounds on body temperature, mast cells and oxygen consumption

Chlorpromazine and other phenothiazines not only cause hypothermia and reduction in oxygen consumption in animals but also rupture mast cells. Mast cells[10] are rich in histamine and 5HT,[2] and these compounds are liberated when rupture of mast cells is induced. However, in the rat 5HT and to a lesser extent histamine administered in large doses, caused hypothermia, and this finding must be taken into account in deciding the cause of hypothermia induced by phenothiazines and other compounds. In Table 1 and Fig. 1, the effects of histamine and 5HT on rectal temperature, oxygen consumption and mast cells are shown together with corresponding results for CPZ and other phenothiazines (promethazine, trimeprazine, pipamazine, trifluoperazine and triflupromazine) and the antihistamines mepyramine and diphenhydramine.

In the rat histamine and 5HT in large doses caused slight reduction in rectal temperature with comparable reductions in QO_2, but no observable changes in the mast cells. Rowley and Benditt[11] also found no action on mast cells after 5HT or histamine administration. The hypothermic effect was of short duration, being maximal at 30 min (Fig. 1). On the other hand, chlorpromazine in large doses (35 mg/kg) caused severe and prolonged hypothermia, a marked reduction in QO_2 and rupture of mature Type II mast cells, leaving the perivascular Type I cells apparently intact, confirming the results of Högberg and Uvnas[10] who described mast cell rupture by certain phenothiazine derivatives. Other phenothiazines tested (promethazine, trimeprazine,

pipamazine, trifluoperazine and triflupromazine) also caused moderate to severe hypothermia, reductions in $\dot{Q}O_2$ and mast cell rupture similar to CPZ. Two antihistamines which are not phenothiazine derivatives (viz. mepyramine and diphenhydramine) were also tested. Mepyramine in large doses (60 mg/kg) caused a slight hypothermia, but failed to rupture mast cells; in larger (near lethal) doses of

TABLE 1. EFFECT OF HISTAMINE, 5HT AND ANTIHISTAMINIC COMPOUNDS ON BODY TEMPERATURE, MAST CELL RUPTURE* AND OXYGEN CONSUMPTION IN ADULT RATS

Treatment (number of rats)			Rectal temperature (°C ± s.e.) Initial	Rectal temperature (°C ± s.e.) 2 hr after intraperitoneal injection	Mast cell rupture	Percentage reduction in $\dot{Q}O_2$ (%)†
Histamine	66 mg/kg	(6)	37·2 ± 0·3	37·1 ± 0·1	intact	20
5HT	15 mg/kg	(6)	37·5 ± 0·2	35·7 ± 0·3	intact	8
CPZ	35 mg/kg	(6)	37·6 ± 0·1	30·8 ± 0·1	ruptured	43
		(6)	37·6 ± 0·2	30·6 ± 0·4	ruptured	43
		(6)	37·6 ± 0·2	29·9 ± 0·4	ruptured	34
		(6)	38·1 ± 0·2	32·4 ± 0·4	ruptured	—
		(5)	38·7 ± 0·2	33·0 ± 0·2	ruptured	—
		(5)	37·6 ± 0·3	32·7 ± 0·6	ruptured	—
		(6)	37·9 ± 0·1	31·5 ± 0·4	ruptured	—
		(6)	38·4 ± 0·2	31·8 ± 0·4	ruptured	35
		(6)	37·5 ± 0·2	28·9 ± 0·3	ruptured	—
		(6)	38·1 ± 0·1	32·9 ± 0·7	ruptured	48
Promethazine	35 mg/kg	(6)	37·4 ± 0·2	33·6 ± 0·3	ruptured	20
		(6)	37·8 ± 0·2	31·8 ± 0·5	ruptured	33
Trimeprazine	25 mg/kg	(3)	37·5 ± 0·5	32·9 ± 0·1	ruptured	—
	15 mg/kg	(6)	37·8 ± 0·1	35·6 ± 0·3	ruptured	7
Pipamazine	25 mg/kg	(6)	38·5 ± 0·3	33·2 ± 0·4	ruptured	—
Trifluoperazine	8 mg/kg	(6)	37·2 ± 0·2	35·6 ± 0·2	ruptured	33
Triflupromazine	25 mg/kg	(6)	37·7 ± 0·2	35·0 ± 0·3	ruptured	9
Mepyramine	60 mg/kg	(6)	36·7 ± 0·2	37·0 ± 0·2	intact	—
	80 mg/kg	(6)	38·1 ± 0·2	36·3 ± 0·2	intact	—
Diphenhydramine	60 mg/kg	(6)	38·6 ± 0·2	34·4 ± 0·4	scattered ruptured	—

* Rupture of mast cells (Riley Type II) in mesentery determined at 2 hr after injection.
† $\dot{Q}O_2$ for control rats was 0·208 ± 0·022 (s.d.) ml O_2/100 g rat per min.
$\dot{Q}O_2$ measured for 30 min before and after injection of drugs.

80 mg/kg, a moderate hypothermia resulted (Fig. 1), but still no rupture of mast cells was observed. Diphenhydramine caused a moderate lowering of rectal temperature and scattered rupture of mast cells in agreement with *in vitro* findings of Arunlakshana.[12] Therefore it appears that the weak to moderate hypothermic action of histamine and 5HT were not associated with mast cell rupture and apparently not dependant on the latter. On the other hand the profound hypothermic effect of CPZ and other phenothiazines tested, and that of diphenhydramine, were accompanied by rupture of mast cells.

Vol. 1
1961

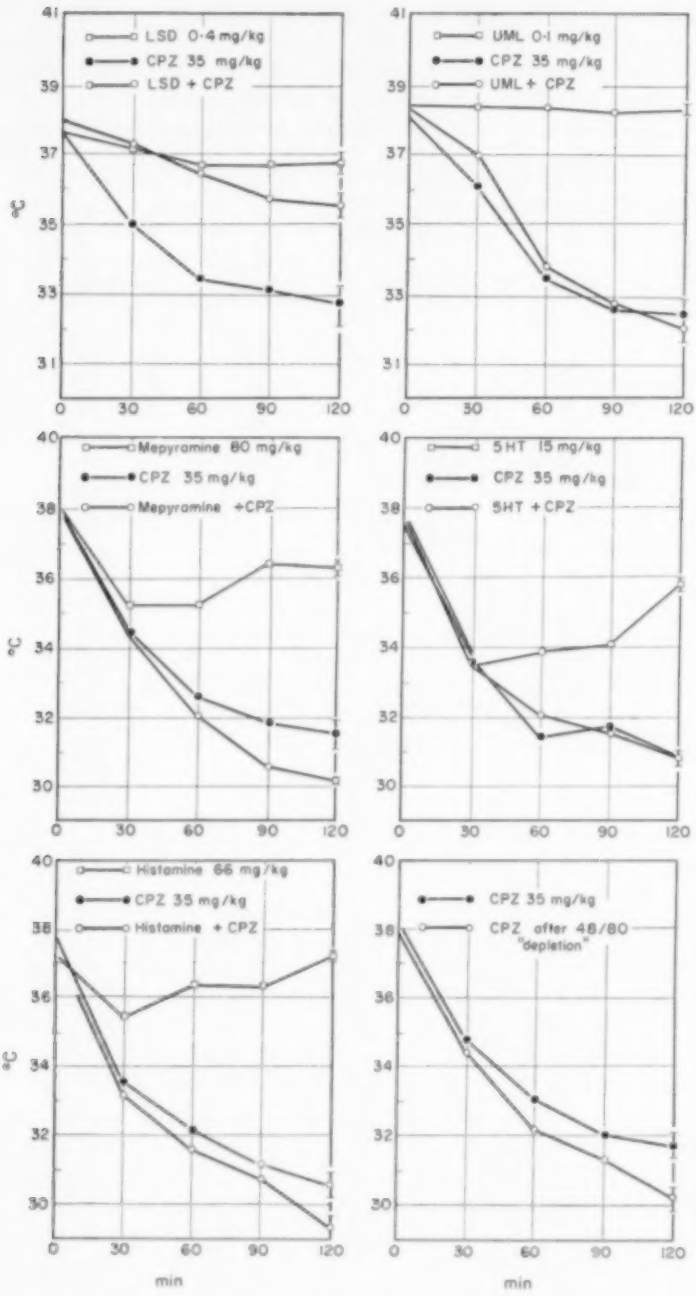


FIG. 1. Effect of CPZ and other compounds on rectal temperature of rats, both singly and in combination. Vertical lines at 120 min denote standard errors.

Since reserpine is particularly associated with the release and depletion of tissue 5HT, and compound 48/80 with corresponding effects on tissue histamine, these compounds were also examined for their effect on rectal temperature, QO_2 and mast cells in rats (Figs. 2 and 3). Reserpine caused a progressive fall in rectal temperature for 18–20 hr after injection (Fig. 2) with a marked reduction in QO_2 but did not cause

TABLE 2. HISTAMINE CONTENT OF RAT MESENTERIES AFTER THE ADMINISTRATION OF SOME PHENOTHIAZINE DERIVATIVES, COMPOUND 48/80, MEPYRAMINE AND RESERPINE

Treatment (number of rats)			Time of extraction after injection (hr)	Mean histamine content in μg/g tissue ± s.e.
Controls (saline)		(7)	24	10·9 ± 1·5
Compound 48/80	1 mg/kg	(6)	24	2·9 ± 0·8
Mepyramine	35 mg/kg	(6)	24	10·4 ± 0·8
CPZ	35 mg/kg	(6)	24	5·7 ± 1·0
Promethazine	35 mg/kg	(6)	24	5·6 ± 0·3
Trimeprazine	25 mg/kg	(6)	24	4·3 ± 0·4
Reserpine	10 mg/kg	(6)	16	8·8 ± 1·0
Reserpine	10 mg/kg	(6)	40	7·4 ± 0·9
Reserpine and CPZ		(6)	16 + 24	6·5 ± 0·7

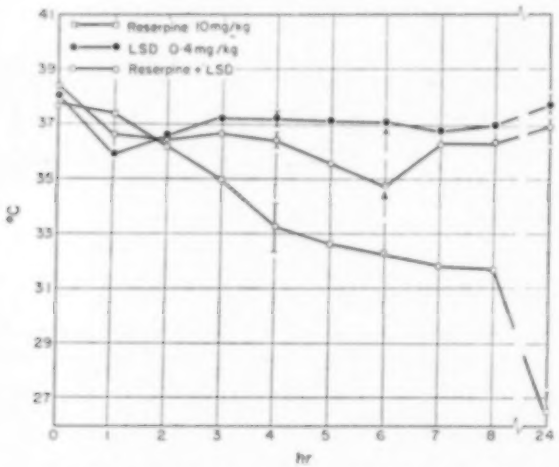


FIG. 2. Rectal temperature of rats treated with reserpine and LSD, singly or in combination. Arrows mark second intraperitoneal injection of a dose of 0·2 mg/kg LSD.

observable rupture of mast cells, although the latter appeared swollen and irregular 16 hr after injection. Compound 48/80 in single doses of 200 μg had a marked hypothermic action (Fig. 3) with a 30 per cent reduction in QO_2, and ruptured all Type II mast cells and most Type I mast cells. At the conclusion of a subacute course of administration of 48/80, the rectal temperature and QO_2 of treated animals did not differ from those of control animals, but only isolated mast cells were found in the mesenteries and subcutaneous tissues (Tables 4 and 5). It follows that a positive association of mast cell rupture and hypothermia holds for compound 48/80 but not for reserpine.

Two hours after the administration of the 5HT antagonists, BOL, UML and LSD there was no significant hypothermia and there were no observable effects on mast cells (Table 3, Fig. 1).

A hallucinogenic agent JB 329, which is an acetylcholine antagonist but has no anti-5HT or antihistamine action,[13] had no action on mast cells and produced only very slight hypothermia (Table 3).

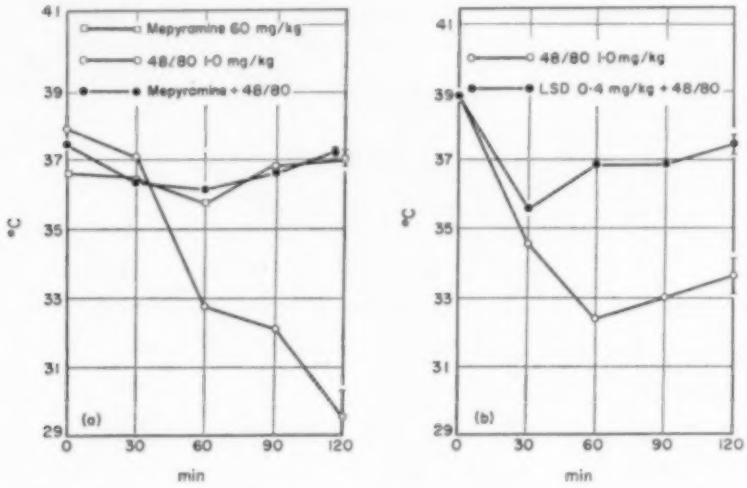


FIG. 3. Effect of (a) mepyramine and (b) LSD on rectal temperature of rats and on the hypothermic action of compound 48/80.

Histamine content of mesentery

Twenty-four hours after the administration of the phenothiazine derivatives, CPZ, promethazine and trimeprazine, assays of rat mesentery showed significant reductions in histamine content to approximately 50 per cent of control values (Table 2). The antihistamine mepyramine did not, however, significantly reduce tissue histamine. Twenty-four hours after administration of a single dose of compound 48/80 (200 μg) there was a great reduction in tissue histamine. Reserpine caused a slight but not significant reduction at 16 hr after injection but at 40 hr the reduction (30 per cent) was significant ($0{\cdot}05 > P > 0{\cdot}01$). It is concluded that whilst hypothermia may be associated with histamine release, drugs which cause the greatest fall in body temperature (and QO_2), e.g. reserpine, CPZ, may be associated with moderate to slight reductions in tissue histamine, whilst agents causing less severe hypothermia (e.g. 48/80) may cause profound reductions in tissue histamine.

Effect of amine antagonists on the actions of CPZ

Since CPZ causes mast cell rupture, release of tissue histamine and also 5HT, the effects of combining the administration of CPZ with antihistamines and anti-5HT drugs were examined, to test further the hypothesis that hypothermia may result secondarily from release either of endogenous histamine or 5HT from mast cells. Conversely the combination of CPZ and either histamine or 5HT was also examined to determine if the effects were additive.

Vol. 7
1961

BOL and UML 491 are peripheral 5HT antagonists but are not hallucinogenic. Pretreatment of animals with BOL in a dose which has been reported to prevent the action of endogenous 5HT[14] failed to modify CPZ induced hypothermia and mast cell rupture (Table 3, Fig. 1). UML 491 gave a similar result. LSD, which is a peripheral 5HT antagonist, but also a hallucinogen, markedly reduced the hypothermic action

TABLE 3. EFFECT OF PRETREATMENT* WITH HISTAMINE, 5HT AND AMINE ANTAGONISTS ON THE HYPOTHERMIA, MAST CELL RUPTURE AND $\dot{Q}O_2$ REDUCTION DUE TO CPZ (35 mg/kg) IN ADULT RATS

Treatment (number of rats)		Rectal temperature (°C ± s.e.) Initial	2 hr after CPZ	Mast cell rupture	% reduction in $\dot{Q}O_2$
CPZ	(6)	37·6 ± 0·2	30·6 ± 0·4	ruptured	43
Histamine 66 mg/kg	(6)	37·2 ± 0·3	37·1 ± 0·1	intact	20
Histamine and CPZ	(6)	37·7 ± 0·2	29·2 ± 0·4	ruptured	44
CPZ	(6)	37·6 ± 0·1	30·8 ± 0·1	ruptured	43
5HT 15 mg/kg	(6)	37·5 ± 0·2	35·7 ± 0·3	intact	8
5HT and CPZ	(6)	37·5 ± 0·2	30·6 ± 0·5	many intact	41
CPZ	(6)	38·1 ± 0·1	32·9 ± 0·7	ruptured	—
5HT 15 mg/kg	(6)	38·3 ± 0·2	35·1 ± 0·5	intact	—
5HT and CPZ	(6)	38·2 ± 0·2	31·4 ± 0·6	many intact	—
CPZ	(6)	37·9 ± 0·1	31·6 ± 0·4	ruptured	—
Mepyramine 80 mg/kg	(6)	38·1 ± 0·2	36·3 ± 0·2	intact	—
Mepyramine and CPZ	(6)	37·8 ± 0·1	30·0 ± 0·2	ruptured	—
CPZ	(6)	37·6 ± 0·2	29·9 ± 0·4	ruptured	34
BOL 3 mg/kg	(6)	37·4 ± 0·2	36·6 ± 0·3	intact	20
BOL and CPZ	(6)	36·9 ± 0·3	29·6 ± 0·6	ruptured	40
CPZ	(6)	38·1 ± 0·2	32·4 ± 0·4	ruptured	—
UML 491 0·1 mg/kg	(6)	38·4 ± 0·2	38·3 ± 0·1	intact	—
UML 491 and CPZ	(6)	38·3 ± 0·2	32·0 ± 0·5	ruptured	—
CPZ	(6)	37·6 ± 0·3	32·7 ± 0·6	ruptured	—
LSD 0·4 mg/kg	(6)	37·6 ± 0·3	36·7 ± 0·4	intact	—
LSD and CPZ	(6)	38·0 ± 0·3	35·5 ± 0·4	ruptured	—
CPZ	(5)	37·9 ± 0·2	33·1 ± 0·5	ruptured	—
JB 329 10 mg/kg	(5)	37·9 ± 0·2	36·4 ± 0·2	intact	—
JB 329 and CPZ	(5)	37·9 ± 0·2	32·1 ± 0·6	ruptured	—

* Compounds injected 5 min before the injection of CPZ.

of CPZ in rats (Fig. 1), as previously reported for mice,[15] but did not modify the mast cell rupture due to CPZ. Similarly LSD reduced the hypothermic action of reserpine in rats (Fig. 2), a finding also reported by Parkes[15] for mice. Another hallucinogen JB 329, which produced very slight hypothermia in rats had no effect on CPZ hypothermia or mast cell rupture. The antihistamine mepyramine, which had little hypothermic action *per se*, and which has been reported to be a most specific antihistamine with little anti-5HT activity[16] failed to modify CPZ induced hypothermia and mast cell rupture. Neither histamine nor 5HT in large doses, modified the effects of CPZ on body temperature and $\dot{Q}O_2$, but whilst histamine also failed to reduce the mast cell response to CPZ, 5HT caused a considerable reduction in the degree of mast cell rupture observed 2 hr after CPZ administration.

Vol. 7
1961

Effect of mepyramine and LSD on the action of compound 48/80

Since the specific antihistamine mepyramine failed to reduce either CPZ induced hypothermia or mast cell rupture, its effect against compound 48/80 was determined. Whilst mepyramine markedly reduced the hypothermia resulting from 48/80 (Fig. 3a) it did not affect the mast cell rupture caused by this substance. LSD also reduced the

TABLE 4. EFFECT OF AMINE DEPLETION BY SUBACUTE TREATMENT WITH 48/80* ON RECTAL TEMPERATURES, MAST CELL RUPTURE AND OXYGEN CONSUMPTION DUE TO THE PHENOTHIAZINES, CPZ (35 MG/KG), PROMETHAZINE (35 MG/KG) AND TRIMEPRAZINE (15 MG/KG)

Treatment (number of rats)	Rectal Temperature (°C ± s.e.)		Mast cell rupture	% reduction in QO_2
	Initial	2 hr after injection of phenothiazine derivative		
CPZ (6)	38·4 ± 0·2	31·8 ± 0·4	ruptured	35
48/80 (subacute depletion) and CPZ (6)	37·8 ± 0·2	30·2 ± 0·4	most mast cells disappeared	43
Promethazine (6)	37·4 ± 0·2	33·6 ± 0·3	ruptured	45
48/80 (subacute depletion) and Promethazine (6)	37·2 ± 0·1	33·2 ± 0·6	most mast cells disappeared	42
Trimeprazine (6)	38·8 ± 0·1	35·6 ± 0·3	ruptured	7
48/80 (subacute depletion) and Trimeprazine (6)	37·7 ± 0·1	35·2 ± 0·3	most mast cells disappeared	10

* Subacute depletion by 48/80 over 4 days injected twice daily, commencing with 50μg doses on first day and increasing total daily dose by an additional 100 μg per day. Rectal temperatures and oxygen consumptions were unaltered at the end of the depletion period.

hypothermic effect of 48/80, without preventing mast cell disruption (Fig. 3b). It would appear that the hypothermic effect of compound 48/80 is due to the release of both histamine and 5HT.

Effect of amine depletion on the action of CPZ and other compounds

(i) *Compound* 48/80. At the termination of subacute depletion of tissue histamine in rats by 48/80, as described under Methods, the rectal temperature and oxygen consumption were normal, but most mast cells (including Type I cells) had disappeared. CPZ administered to such depleted rats. caused as great a fall in rectal temperature and oxygen consumption as in control animals. Similarly the effect of promethazine and trimeprazine was not reduced by pretreatment with 48/80 (Table 4, Fig. 1).

(ii) *Reserpine*. Sixteen hours after administration of reserpine the rectal temperatures and oxygen consumptions of rats were greatly reduced and treatment with CPZ at this stage did not cause any further reduction in these values. The mast cells in reserpinized rats were swollen but intact and suffered less rupture as a result of administration of CPZ than in control animals (Table 5).

(iii) 48/80 *and reserpine*. In one experiment rats pretreated with 48/80 for 4 days were given reserpine. Sixteen hours later, the falls in rectal temperature and oxygen

TABLE 5. EFFECT OF CPZ (35 MG/KG) ON RECTAL TEMPERATURE, MAST CELLS AND OXYGEN CONSUMPTION IN RATS PRETREATED WITH RESERPINE (10 MG/KG INTRAPERITONEAL INJECTION 16 HR BEFORE CPZ) AND IN RATS PRETREATED WITH BOTH 48/80 (SUBACUTE DEPLETION) AND RESERPINE

Treatment (number of rats)		Rectal temperature C° ± s.e.			Mast cell rupture	% reduction QO_2
		Before reserpine	16 hr after reserpine	2 hr after CPZ (or 18 hr after reserpine)		
Reserpine only	(9)	—	32·0 ± 1·6	(31·0 ± 1·1)	swollen but intact	34*
CPZ only	(6)	—	38·1 ± 0·3	32·9 ± 0·7	ruptured	48
Reserpine and CPZ		—	32·3 ± 1·1	33·4 ± 1·0	many intact	30
CPZ only	(6)		37·5 ± 0·2	28·9 ± 0·3	ruptured	—
Reserpine and CPZ	(6)	37·9 ± 0·1	30·9 ± 1·2	29·9 ± 1·0	many intact	—
Reserpine only	(6)	37·9 ± 0·2	32·9 ± 0·5	(32·7 ± 1·0)	—	27*
48/80 and reserpine	(6)	38·0 ± 0·2	32·4 ± 0·4	(33·4 ± 0·4)	—	35*
48/80, reserpine and CPZ	(6)	38·0 ± 0·2	33·4 ± 0·4	34·4 ± 0·4	—	—

* QO_2 measured 18 hr after reserpine injections.

consumption were similar to those in controls treated with reserpine alone. CPZ given to rats already treated with both 48/80 and reserpine, did not cause a further fall in body temperature. In fact the temperature which was gradually returning to normal at the time of CPZ injection, maintained its upward trend (Table 5).

Effect of cyanide and 2:4-DNP on the action of CPZ

In an initial experiment NaCN in doses of 1 mg/kg failed to reduce the hypothermia and mast cell response elicited by CPZ (35 mg/kg) (Table 6). Since this result did

TABLE 6. EFFECT OF METABOLIC INHIBITORS ON THE FALL IN RECTAL TEMPERATURE AND MAST CELL RUPTURE DUE TO CPZ IN ADULT RATS*

Treatment			Rectal temperature (°C ± s.e.)		Mast cell rupture
			Initial	2 hr after injection(s)	
CPZ	35 mg/kg	(1)	38·0	32·5	ruptured
DNP	15 mg/kg		38·7 ± 0·2	39·9 ± 0·4†	intact
DNP and CPZ		(2)	37·5 ± 0·5	38·0 ± 1·0	ruptured
CPZ	35 mg/kg	(5)	38·7 ± 0·2	33·0 ± 0·2	ruptured
NaCN	1 mg/kg	(5)	39·0 ± 0·2	37·7 ± 0·1†	intact
NaCN and CPZ		(5)	38·2 ± 0·1	32·2 ± 0·4	ruptured
CPZ	10 mg/kg	(5)	36·8 ± 0·1	35·5 ± 0·5	ruptured
KCN	0·66 mg/kg	(4)	37·5 ± 0·2	38·0 ± 0·1	intact
KCN and CPZ			37·1 ± 0·2	34·7 ± 0·4	ruptured

* Albino rats were used in this experiment to conform with that of Le Blanc.[5]

† The doses of DNP and NaCN used caused an elevation of 50 per cent and a reduction of 35 per cent in QO_2, respectively.

Vol. 7
1961

not agree with the findings of Le Blanc[5] the experiment was repeated using the conditions described by the latter. However, in our experiments KCN (0·66 mg/kg) failed to reduce either the hypothermic effect or mast cell response due to CPZ (10 mg/kg).

2 : 4-Dinitrophenol (DNP) raised rectal temperatures in rats and increased oxygen consumption, but was without observable effect on mast cells. The administration of DNP prior to CPZ prevented the hypothermic action of CPZ but not the mast cell rupture.

Effect of refrigeration on rat mast cells in vivo

The mast cells in anaesthetized rats subjected to refrigeration for from 2 to 3 hr at − 10 °C, were intact, although rectal temperatures had fallen to 18–20 °C.

DISCUSSION

From the results obtained in this series of experiments it appears that the property of rupturing mast cells in the rat, with the resultant release of amines, is not related to the hypothermic action of CPZ and related compounds studied. Antagonists of both histamine and 5HT even in massive doses failed to reduce the hypothermia produced by CPZ in agreement with the results of Le Blanc and Rosenberg.[17] However, the lowering of rectal temperature due to compound 48/80 was reduced by large doses of a specific antihistamine (mepyramine) without altering the mast cell rupture. Thus it appears that only compound 48/80 produces hypothermia mediated through its amine releasing action. This is supported by the finding that a large dose of 48/80 administered to rats previously treated with this substance to cause disappearance of most tissue mast cells, has a much less effect on body temperature. Prior depletion of mast cell amines by compound 48/80 did not modify the hypothermia caused by CPZ, promethazine or trimeprazine—this suggests a dissociation of hypothermia from histamine release for these latter substances.

In these experiments, contrary to the findings of Le Blanc,[5] cyanide was ineffective against the temperature and mast cell effects of CPZ. However, DNP antagonized CPZ induced hypothermia whilst not preventing mast cell rupture. This latter result showing a lack of correlation between amine release and temperature effects is perhaps better shown with LSD, since DNP itself raises rat rectal temperature and thus could inhibit induced hypothermia by direct non-specific antagonism. In rats LSD itself produced a slight short lasting reduction in rectal temperature yet was effective in reducing the hypothermia caused by CPZ. Although LSD was apparently without effect on mast cells, and did not modify the mast cell response to CPZ, its antagonism of hypothermia produced by CPZ suggests that this effect of LSD may be due to: (1) its anti-5HT action against 5HT liberated peripherally; (2) its anti-5HT action against centrally liberated 5HT or (3) "hallucinogenic activity" possibly unrelated to 5HT. Since 5HT antagonists with much less central action, viz. BOL and UML 491, failed to reduce the effects of CPZ on body temperature, QO_2 and mast cells, the first proposition is not supported. On the other hand, the hallucinogen JB 329, which has marked peripheral anticholinergic action[13] but weak antihistamine and anti-5HT activity did not reduce either CPZ hypothermia or mast cell rupture. This makes the second proposition most likely, viz. that the hypothermic effect of CPZ is mediated through the release of central 5HT. Further, after administration of reserpine which depletes catechol amines and 5HT, CPZ exerted no further hypothermic action,

although it is difficult to judge quantitative effects in such reserpinized animals which are already hypothermic. However, it does appear that brain catechol amines and/or 5HT are involved in the hypothermic action although the mechanism is obscure. As Lessin and Parkes[18] suggested there does appear to be an association between c.n.s. depressant action and hypothermia, at least amongst the antihistamines studied here and for reserpine. However, it is more difficult to suggest which action precedes the other, although a logical sequence may be primarily a c.n.s. depressant action, consequent loss of motor activity and decreased heat production resulting in hypothermia.[19] In all cases reductions in temperature were accompanied by similar degrees of reduction in oxygen consumption. The mast cell rupture caused by some antihistamines would then be merely another of the many "side effects" of this group of compounds seen in varying degrees from mepyramine with no action on mast cells to the phenothiazine derivatives which disperse the granules in almost all Type II mast cells.

Certainly hypothermia itself does not alter the mast cells, as seen in rats which were subjected to refrigeration and whose rectal temperatures were as low as 18 °C. There seems to be a relationship between hypothermia, mast cell rupture and histamine release caused by compound 48/80 since injected histamine caused hypothermia, and this hypothermia as well as that due to compound 48/80 was antagonized by the specific antihistamine mepyramine, without preventing mast cell rupture caused by 48/80. This latter finding is at variance with the statements of Riley[20] that mepyramine antagonizes mast cell rupture due to both chemical histamine liberators and anaphylotoxins.

Acknowledgements—We wish to thank Sandoz Australia Ltd., for gifts of LSD, BOL and UML, Wellcome Foundation, London, for supplies of Compound 48/80 and Dr. S. Gershon for a gift of JB 329. Miss S. Thomas is thanked for statistical help; Mr. A. Rowlatt, Miss K. Ladner and Miss H. Hutchings gave excellent technical assistance.

REFERENCES

1. J. F. Riley and G. B. West, *J. Path. Bact.* **69**, 269 (1955).
2. G. P. Lewis, *5-Hydroxytryptamine* (Edited G. P. Lewis). Pergamon Press, London (1958).
3. J. Le Blanc, *Proc. Soc. Exp. Biol., N.Y.* **97**, 238 (1958).
4. L. C. U. Junqueira and B. Beiguelman, *Texas Rep. Biol. Med.* **13**, 69 (1955).
5. J. Le Blanc, *Proc. Soc. Exp. Biol., N.Y.* **100**, 635 (1959).
6. H. A. S. van den Brenk, *Brit. J. Exp. Path.* **39**, 356 (1958).
7. J. F. Riley, *J. Path. Bact.* **65**, 461 (1953).
8. H. A. S. van den Brenk and M. Haas, *Int. J. Radiobiol.* In press.
9. C. F. Code and F. C. McIntire, *Methods of Biochemical Analysis* (Edited by D. Glick) Vol. III, p. 49. Interscience Publishers, London (1956).
10. B. Högberg and B. Uvnäs, *Acta Chem. Scand.* **11**, 1092 (1957).
11. D. A. Rowley and E. P. Benditt, *J. Exp. Med.* **103**, 399 (1956).
12. O. Arunlakshana, *J. Physiol.* **119**, 47 (1953).
13. S. Gershon, *Nature, Lond.* **186**, 1073 (1960).
14. J. R. Parrat and G. B. West, *Brit. J. Pharmacol* **13**, 65 (1958).
15. M. W. Parkes, *5-Hydroxytryptamine* (Edited by G. P. Lewis) p. 88. Pergamon Press, London (1958).
16. E. W. Packman, G. V. Rossi and J. W. E. Harrisson, *J. Pharm, Lond.* **5**, 301 (1953).
17. J. Le Blanc and Rosenberg, *Proc. Soc. Exp. Biol., N.Y.*, **97**, 95 (1958).
18. A. W. Lessin and M. W. Parkes, *Brit. J. Pharmacol.* **12**, 245 (1957).
19. D. R. Wood, *Brit. J. Pharmacol.* **5**, 195 (1950).
20. J. F. Riley, *The Mast Cells*. Livingstone, Edinburgh and London (1959).

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 47–54. Pergamon Press Ltd., Printed in Great Britain.

THE ENZYMIC DEPHOSPHORYLATION AND OXIDATION OF PSILOCYBIN AND PSCILOCIN BY MAMMALIAN TISSUE HOMOGENATES*

A. HORITA and L. J. WEBER

Department of Pharmacology, School of Medicine, University of Washington
Seattle 5, Washington

(*Received* 14 *December* 1960)

Abstract—Incubation of psilocybin with rat kidney homogenates caused a rapid liberation of its —OH congener, psilocin, through the action of alkaline phosphatase. The psilocin thus formed underwent further degradation to form a blue-colored product. This last step appeared to be catalyzed by an oxidative enzyme which was cyanide-sensitive, but not to β-phenyl*iso*propylhydrazine, a monoamine oxidase inhibitor; its optimal activity was at pH 9·0. In many respects it resembles the characteristics of a phenolase type enzyme. The distribution of the phosphatase and oxidase enzymes in a number of tissues from several species of animals was also investigated. Psilocybin-dephosphorylating activity was highest in the rat and mouse kidney and the mucosa of the small intestine of guinea pig and rabbit. Oxidase activity was highest in the heart of all species and in the kidney of the rat and mouse. These experiments indicate that possibly in the intact animal psilocybin is rapidly dephosphorylated and is pharmacologically active as psilocin, while the duration of the effect might be controlled by the oxidation of the latter compound to an *o*-quinone type of structure.

COMPOUNDS possessing indole structures have gained much interest in recent years because of their hallucinogenic actions in man. A considerable number of such compounds are now known and, among these, two stand out because of their unique chemical structures. These are the 4-substituted indoleamines, psilocybin (4-phosphoryl-N: N-dimethyltryptamine) and psilocin (4-hydroxy-N: N-dimethyltryptamine).† Both indoles were first isolated from the mushroom, *Psilocybe mexicana* Heim, and found to be the active hallucinogenic material in this species. The chemistry[1, 2] and various pharmacologic actions[3–5] of these indoles have been described, but little is known as to their metabolism by mammalian tissue systems.

Recent investigations in this laboratory have demonstrated that psilocybin acts as an excellent substrate for the purified alkaline phosphatase enzyme.[6] The dephosphorylation resulted in the liberation of its 4-OH congener, psilocin. The fact that non-specific monophosphoesterases are abundant in various mammalian tissues[7] suggested that possibly a biotransformation of psilocybin to psilocin might occur *in vivo*. This also implied that possibly the active pharmacologic agent after psilocybin administration could be its dephosphorylated congener. The present studies are concerned in part with the *in vitro* dephosphorylation of psilocybin by mammalian

* This study was supported in part by a grant from the U.S. Public Health Service (MY-2435(C2)).

† The psilocybin and psilocin used in this study was generously provided by Mr. Harry Althouse of Sandoz Pharmaceuticals, San Francisco, California.

tissue homogenates. In the course of these studies it was found that not only was psilocybin rapidly dephosphorylated to liberate psilocin, but also the latter compound appeared to undergo further transformation to a blue-colored product. Since the same results were not seen when purified alkaline phosphatase was employed as the enzyme source, it appeared that a second enzyme system was involved. It is the purpose of this report, therefore, to describe some of the characteristics of these two steps in the biotransformation of psilocybin. After some preliminary work it appeared that a dilute kidney homogenate would best serve as the source of the enzymes. The distribution of the phosphatase and the oxidase enzymes was also examined in several species of animals. This was done in order to determine which tissues and species possessed the greatest ability to dephosphorylate and oxidize the 4-substituted indoles. It was considered that by determining their distributions the identification of the oxidase might be aided, especially if it should possess characteristics and distribution patterns similar to those of other known enzymes.

METHODS

Kidneys from male Sprague–Dawley rats were used to prepare the homogenates. The tissue was ground with a Teflon homogenizer in a concentration of 5 per cent with distilled water. All procedures were carried out under ice-cold conditions. With the exception of studies on the effect of pH, a veronal buffer (0·1 M) was employed as the buffer system of the phosphatase and oxidase enzymes. For studies of the pH optimum, a buffer comprised of the following agents was used: Na_2HPO_4 (0·1 M), Tris buffer (0·3 M), β-alanine (0·4 M), and DL-lysine (0·4 M), and adjusted to the proper pH level by the addition of 1·0 N NaOH. This mixture was satisfactory as a buffer in the range of pH from 7 to 10, although it did exert some inhibitory effect on the oxidase system.

The incubation mixture for the determination of phosphatase activity contained the following: 1 ml of kidney homogenate, 1 ml of veronal buffer (0·1 M), 0·3 ml of KCN (0·01 M), and 1 ml of a solution of psilocybin (2 μmoles/ml) in distilled water. The flasks were incubated at 37 °C in a constant temperature Dubnoff-type shaker for the designated times, removed and heated in a boiling water bath for from 3 to 4 min to stop the reaction. Two milliliters of the mixture were then transferred to a shaking bottle containing 15 ml of *n*-butanol, 1 ml of borate buffer (pH 10), and 2 g of solid NaCl. The remainder of the procedure was similar to the method for the colorimetric analysis of serotonin described by Udenfriend *et al.*[5] The final colors of the psilocin were determined spectrophotometically at 430 mμ, as described earlier by Horita and Weber.[6] Known amounts of psilocin were carried throughout the entire extraction procedure and used as standards. As indicated previously, a linear relationship between optical density and concentration was found between the range of from 0·02 to 1·0 μmoles of psilocin per ml.

The oxidase activity was measured both by the disappearance of psilocin and grossly by observation of the formation of a blue color in the incubation mixture. Flasks were arranged similarly as in the phosphatase study and consisted of the following: 1 ml of kidney homogenate, 1 ml of veronal buffer, and 1 ml of a solution of psilocin (from 2 to 4 μmoles/ml). The final pH of the mixture was 8·8. The substrate was added to the flask after equilibration to 37 °C. Two milliliters of the incubation mixture were withdrawn after the appropriate time and transferred to the shaking bottles containing

Vol. 7
1961

the *n*-butanol, borate buffer and solid NaCl. The remainder of the procedure for the determination of psilocin is as described above. In determining the species variation of the two enzymes involved, the rat, mouse, guinea pig and rabbit were used as the experimental animals. Brain, liver, kidney, heart and intestinal mucosa were compared for their abilities (1) to dephosphorylate psilocybin and (2) to oxidize psilocin to the blue-colored product. These processes were measured by psilocin liberation from psilocybin and disappearance of psilocin, respectively. Incubations were made as described above, using the 5 per cent homogenate, 2 μmoles of the substrate; the vessels were shaken for a period of 20 min. The remainder of the extraction and analysis was identical to that described above.

RESULTS

The dephosphorylation of psilocybin by a 5 per cent rat kidney homogenate proceeds at an extremely rapid rate. As can be seen in Fig. 1, an essentially maximal

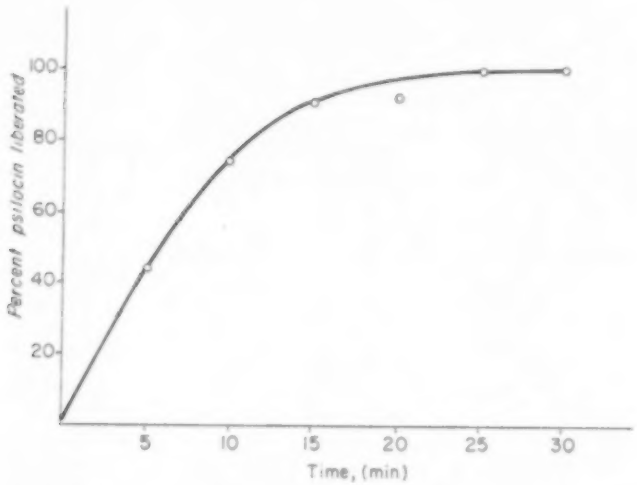


FIG. 1. The rate of psilocin liberation from psilocybin by rat kidney homogenate. Psilocybin (2 μmoles) was incubated with 1 ml of kidney homogenate (5 per cent), 10^{-3} M KCN, and 0·1 M veronal buffer. Final volume, 3·3 ml; pH 8·8. Temperature 37 °C.

liberation of psilocin was accomplished within 15 min of incubation, indicating that the alkaline phosphatase activity in kidney tissue was relatively high. The effect of altering pH on the dephosphorylation of psilocybin was also investigated. Fig. 2 shows a single peak of pH 9·0, indicating the optimal pH for the activity of alkaline phosphatase. Even at the physiological pH of 7·4 a considerable amount of dephosphorylation was evident.

In all of the experiments with the phosphatase enzyme, KCN was added to the incubation mixture (final concentration, 10^{-3} M). In the absence of KCN the total amount of psilocin formation was not measurable, because of its rapid degradation by a cyanide-sensitive enzyme system which will be described below. KCN at 10^{-3} M

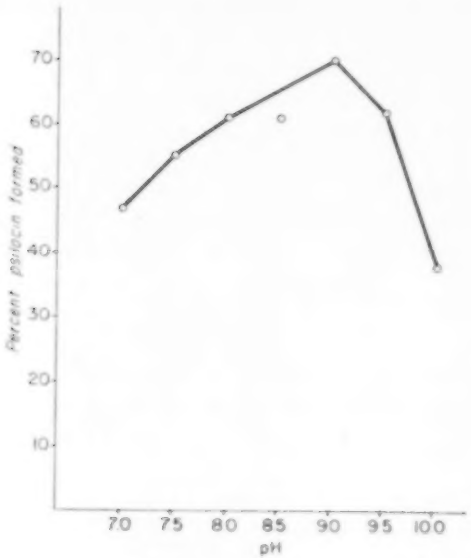


FIG. 2. The effect of pH on psilocin liberation from psilocybin by rat kidney homogenate. Psilocybin (2 μmoles) was incubated with 1 ml of kidney homogenate (5 per cent), 10^{-3} M KCN, and buffer (described in text) for 40 min at 37 °C.

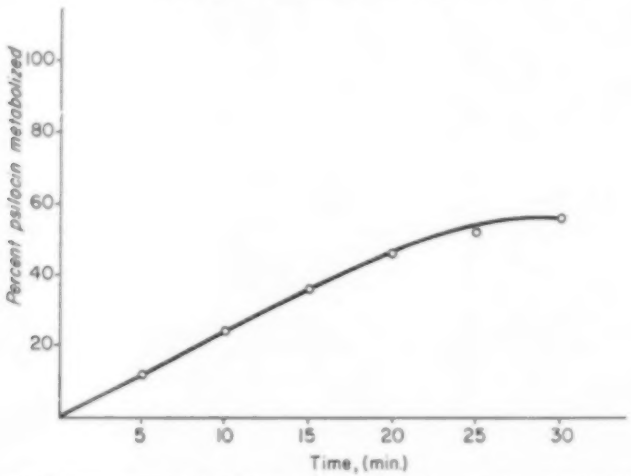


FIG. 3. The rate of disappearance of psilocin in rat kidney homogenate. Psilocin (2 μmoles) was incubated with 1 ml of kidney homogenate (5 per cent) in 0·1 M veronal buffer. Final volume, 3 ml; pH 8·8. Temperature 37 °C.

was completely effective in protecting the psilocin from degradation, but did not influence the phosphatase activity.

Incubation of psilocin with rat kidney homogenate in veronal buffer (final pH, 8·8) resulted in psilocin disappearance at a linear rate over a 25-min period (Fig. 3). Beyond this period, the rate of breakdown decreased indicating that a submaximal

substrate concentration had been reached. The disappearance of the psilocin was accompanied by the development of a blue color in the incubation mixture. When higher concentrations of psilocin and kidney homogenate were used the development of the blue color was extremely rapid and intense, reaching a dark blue within a matter of minutes. The addition of KCN (final concentration 10^{-3} M) completely prevented the appearance of the blue color, as well as the disappearance of psilocin. When the reaction was carried under a nitrogen atmosphere similar results were found as with KCN, indicating that the degradation of psilocin by rat kidney homogenate involved an aerobic oxidative process. The monoamine oxidase inhibitor, β-phenyl*iso*propylhydrazine, exerted no influence on the psilocin breakdown reaction.

The pH optimum of this oxidative reaction was determined. As shown in Fig. 4, the reaction progressed with increased pH until the optimum of pH 9·0 was reached.

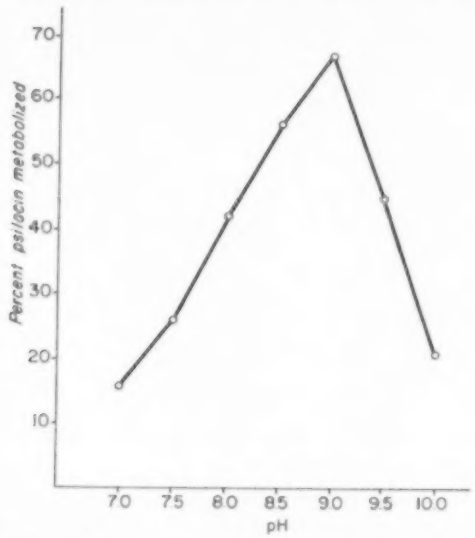


FIG. 4. The effect of pH on the disappearance of psilocin in rat kidney homogenate. Psilocin (2 μmoles) was incubated with 1 ml of kidney homogenate (5 per cent) in buffer (described in text) for 40 min at 37 °C.

Beyond this point the activity fell rapidly. Like the phosphatase reaction, the oxidation of psilocin also occurred to some extent at the pH of 7·4.

The distribution of the phosphatase and oxidase enzymes acting upon psilocybin and psilocin, respectively, in the various species of animals is tabulated in Table 1. All values are expressed as per cent substrate metabolism (psilocybin by phosphatase, psilocin by the oxidase). A noticeable resemblance is apparent in the distribution of the enzymes in the various animals. This is especially true in the mouse and rat, in which case all of the activities in the tissues investigated were quite similar. Kidney phosphatase in these two species were especially high, while in the guinea pig and rabbit the mucosa of the small intestine displayed the highest phosphatase activity. Under the conditions of these experiments these tissues were capable of dephosphorylating essentially the entire amount of psilocybin present. In all animals the liver and heart were low in their ability to dephosphorylate psilocybin.

The extent of oxidation of psilocin also varies with the species and the source of the enzyme. In all of the species the heart exhibited more oxidase activity than any of the other tissues tested. The rat and mouse kidney also showed high activity. The lowest activity was consistently found in the small intestine.

DISCUSSION

That mammalian tissues can dephosphorylate psilocybin with the liberation of psilocin is evident from the present studies. The rate of dephosphrylation, as shown with the 5 per cent rat kidney homogenate, indicates a high activity of the enzyme in this tissue. Since the reaction occurs at pH levels in the physiological range, as well as at the optimum of pH 9, it is most probable that psilocybin dephosphorylation

TABLE 1. ALKALINE PHOSPHATASE AND PSILOCIN-OXIDIZING ACTIVITIES OF VARIOUS TISSUE HOMOGENATES FROM FOUR SPECIES OF ANIMALS

Figures are expressed as per cent substrate metabolism (psilocybin by phosphatase, psilocin by oxidase). Figures represent the mean values of from three to four determinations.

	Rat		Mouse		Guinea pig		Rabbit	
Enzyme / Tissue	Phos-phatase	Oxidase	Phos-phatase	Oxidase	Phos-phatase	Oxidase	Phos-phatase	Oxidase
Kidney	99	57	95	57	65	27	34	39
Heart	14	61	13	65	7	64	4	68
Brain	14	28	28	32	20	25	5	30
Liver	3	35	1	37	3	25	5	8
Small intestine	13	17	10	14	88	15	96	9

occurs *in vivo* as well as *in vitro*. Evidence that this takes place in the intact rat has been observed (unpublished observations).

The rapid transformation of psilocybin to psilocin raises the question as to whether the former compound plays any role in exerting the central nervous system effects which are seen after its administration to either man or animals. It would appear likely that the active substance is its dephosphorylated congener, psilocin, since it possesses greater lipid solubility (unpublished observations) and would theoretically pass the blood–brain barrier more readily than psilocybin. It would be interesting to determine whether a specific inhibitor of the phosphatase enzymes would delay or inhibit the actions normally exerted by psilocybin.

The oxidation of the psilocin liberated from the psilocybin by the kidney homogenate was an unexpected finding. In the earlier exploratory experiments kidney homogenates at concentrations as high as 33 per cent were employed for the phosphatase reaction. Upon adding the psilocybin to the incubation mixture a blue color developed within a few minutes, and within 10 min turned into an intense blue-black mixture. When psilocin was incubated in the same manner it also disappeared with the formation of the blue color. With other tissue homogenates, such as the heart, psilocybin did not display the blue color as rapidly as with kidney; however, psilocin was quickly converted to the colored state. It appears that in order to be oxidized to the blue-colored product, psilocybin must first be dephosphorylated to psilocin. The nature of

Vol. 7
1961

this oxidation product is unknown. Recently, Blaschko and Levine[9] described the presence of a hydroxyindole oxidase in the gill plates of *Mytilus edulis*; this enzyme was capable of oxidizing psilocin with the formation of a blue-colored product. The authors suggested that the final product might be of an *o*-quinoid structure. It is probable that the oxidation of psilocin by the *Mytilus* oxidase results in the same product as found with the kidney homogenate, but it is unlikely that the enzymes in the two systems are identical.

The high pH optimum of the rat kidney oxidase system is unusual, and it was first thought that possibly the oxidation of psilocin was a non-enzymatic auto-oxidation similar to that found for DOPA at alkaline pH.[10] However, the fact that a pH optimum peak was present indicated that the reaction was enzymic. No auto-oxidation of psilocin was found when the compound was incubated with veronal buffer at pH 9·0 for the times designated in the section on Results. Incubation with kidney homogenate buffered at pH 11 or above showed almost no breakdown of psilocin. Furthermore, definite differences in activity were found among the various tissues and in the different species findings which suggest that this was not a non-specific auto-oxidation process. Finally, exposing the homogenate to a boiling water bath abolished its enzymic properties.

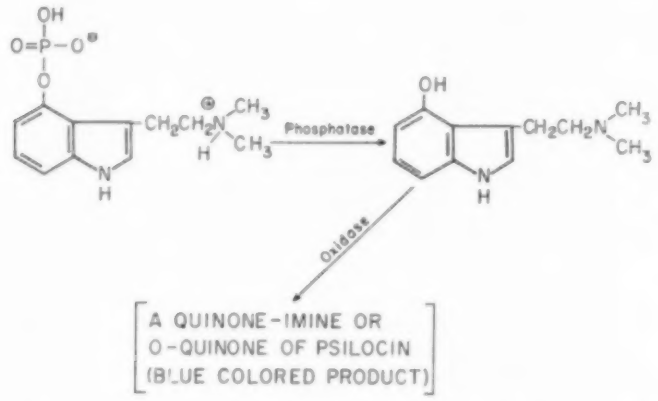


FIG. 5. Diagram representing the metabolic pathway of psilocybin. The structures in brackets indicate hypothetical oxidation products of psilocin.

Preliminary work with possible inhibitors of this oxidase system establishes the fact that monoamine oxidase is not involved. Not only do the optimum conditions differ, but also the potent monoamine oxidase inhibitor, β-phenyl*iso*propylhydrazine, has no influence on the oxidative process. KCN, on the other hand, which does not block monoamine oxidase, was effective in inhibiting the degradation of psilocin. The distribution of the oxidase activity in the various tissues also does not resemble that of monamine oxidase. Generally, the liver is the richest source of monoamine oxidase, and heart and kidney are relatively low; however, the oxidase which is active against psilocin is highest in the heart and kidney, with the liver showing only minimal activity.

Whatever the nature of this oxidative process might be it is evident that homogenates prepared from tissues of various animals are capable of dephosphorylating psilocybin to form psilocin, and the latter further metabolized to form a blue-colored product. In the rat kidney the oxidative process appears to be the rate-limiting step, since it was shown that under the conditions employed, dephosphorylation proceeded at a much faster rate than did the disappearance of psilocin. This does not hold true for all tissues, however, since the findings with preparations like heart homogenate, which possess low phosphatase activity, indicate that this enzyme catalyzes the rate-limiting step. The scheme, Fig. 5, illustrates diagrammatically the pathway of degradation of psilocybin. The structures in brackets are hypothetical, but since the phenolase reaction involves the hydroxylation of the *o*-position of the ring, followed by an *o*-quinone formation,[11] we suggest, as did Blaschko and Levine,[9] that the *o*-quinone of psilocin is responsible for the blue color. Our recent studies with a rabbit heart mitochondria preparation and a purified pig heart cytochrome oxidase indicate that a phenolase-like action of this enzyme may be involved in this oxidation process.

REFERENCES

1. A. HOFFMAN, R. HEIM, A. BRACK, H. KOBEL, A. FREY, H. OTT, TH. PETRIZILKA and F. TROXLER, *Helv. Chim. Acta* **42**, 1557 (1959).
2. F. TROXLER, F. SEEMAN and A. HOFFMAN, *Helv. Chim. Acta* **42**, 2074 (1959).
3. H. WEIDMANN, M. TAESCHLER and H. KONZETT, *Experientia* **14**, 378 (1958).
4. M. MONNIER, *Experientia* **15**, 321 (1959).
5. H. WEIDMANN and A. CERLETTI, *Helv. Physiol. Acta* **18**, 174 (1960).
6. A. HORITA and L. J. WEBER. In press.
7. J. ROCHE, *The Enzymes* (Edited by SUMNER and MYRBACK) Vol. 1, part 1. Academic Press, New York (1950).
8. S. UDENFRIEND, H. WEISSBACH and B. B. BRODIE, *Meth. Biochem. Anal.* **6**, 95 (1958).
9. H. BLASCHKO and W. G. LEVINE, *Biochem. Pharmacol.* **3**, 168 (1960).
10. C. MONDER, J. N. WILLIAMS, JR. and H. A. WAISMAN, *Arch. Biochem. Biophys.* **72**, 255 (1957).
11. H. S. MASON, *Advanc. Enzymol.* **16**, 105 (1955).

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 55–58. Pergamon Press Ltd., Printed in Great Britain.

EXPERIMENTAL BOWEL SHOCK IN RATS*—II
LIVER NUCLEOTIDE CHANGES

O. W. WENDEL† and E. M. BROPHY

Department of Chemistry, Bethany College, Bethany, W.Va., and
Department of Obstetrics and Gynecology, Southwestern Medical College of the University of Texas, Dallas, Tex.

(*Received* 20 *October* 1960)

Abstract—Rats were subjected to a standardized bowel strangulation procedure, and their livers removed, frozen and homogenized in cold perchloric acid. An aliquot of neutralized acid-supernatant fluid was adjusted to pH 8·4 and chromatographed on Dowex-1 chloride. Nucleotide fractions were identified by their ultraviolet absorption spectra.

As compared to control animals, the amount of adenosine triphosphate was found to be decreased in liver homogenates of untreated shocked rats, and, in addition, three unusual peaks were detected. One peak was shown to be hypoxanthine, whereas the other two were chromatographically similar to inosine monophosphate. Medication with neomycin sulfate prevented the formation of these products of catabolism in shocked animals. Unshocked animals medicated with neomycin sulfate had significantly greater quantities of liver adenosine triphosphate than did similarly treated controls.

ALTERATIONS in some chemical constituents of plasma and tissues of shocked animals have been extensively investigated. Biochemical and physiological changes associated with traumatic,[1] endotoxin[2] and bowel obstruction[3] shock, including changes in protein[4, 5] and nitrogenous substances[6, 7] have been reported.

One marked change which occurs following trauma is an increase in inorganic phosphate and a decrease in adenosine triphosphate (ATP‡) in tissue.[8] Animals premedicated with antibiotics prior to experimental bowel obstruction exhibit reduced necrosis of the strangulated segment[9, 10] and increased survival.[11, 12] Rosenbaum *et al.*[13] have shown that medication with aureomycin prior to induction of hemorrhagic shock resulted in the maintenance of control levels of "high energy phosphate" compounds in the liver. Quantitative changes in nucleotide metabolism in the livers of shocked animals have not been reported.

An attempt has been made to quantitate the changes in certain nucleotide concentrations in liver homogenates following bowel shock, and to determine the influence of neomycin premedication. Because chemical and enzymic analyses of various nucleotides yields only limited and indirect information, ion exchange chromatography was applied to the problem.

MATERIALS AND METHODS

Female rats of the Holtzman strain, weighing from 200 to 275 g, were used. All animals were maintained *ad libitum* on Purina laboratory chow and water; those

* This study was supported by grants from the Gans Fund and the Smith, Kline and French Foundation.

† Present address: Sterling-Winthrop Research Institute, Rensselaer, N.Y.

‡ AMP, ADP and ATP refer to adenosine mono-, di- and tri-phosphate, respectively. IMP, IDP and ITP refer to inosine mono-, di- and tri-phosphate, respectively.

animals medicated with antibiotic received 2 g of neomycin sulfate* per kg of body weight by stomach tube daily for 5 days prior to obstructing the bowel segment.

The method for inducing a standardized bowel obstruction which leads to a state of shock, was that described by Wendel *et al.*[3] Control animals were sham operated. For maximum measurable effects the occlusion time was 4 hr. At the time of obstruction release, or 2 hr thereafter, the animals were anesthesized with ether, and the livers removed and dropped into a dry ice–acetone mixture. Tissues from control animals were obtained and treated in a similar manner. The tissues were removed from the freezing mixture, blotted, weighed and pulverized in a chilled steel mortar. The powdered tissue was homogenized in a Waring blender with 3 vols. (30 ml acid to 10 g of tissue) of cold 0·6 N perchloric acid. The mixture was centrifuged in the cold, the supernatant fraction removed, and the residue re-extracted with 1 vol. of cold 0·6 N perchloric acid. The combined supernatant fractions were neutralized in the cold with 5 N KOH to pH 6·5, using phenol red as an internal indicator. The neutralized extract was then cooled to near freezing and the precipitated potassium perchlorate removed by centrifugation. The resulting supernatant material was brought to volume with water and an aliquot (adjusted to pH 8·4 with ammonium hydroxide) taken for chromatographic analysis.

The adenine nucleotides were resolved and separated quantitatively on columns of Dowex-1 in the chloride cycle by the method of Cohn and Carter[14]. In order to resolve the IMP and ADP which come off the column together, the volume of the first eluting solvent (0·003 N HCl) was increased from 100 to 350 ml. To detect IMP, an eluant composed of 0·05 M NaCl–0·01 N HCl was used instead of 0·2 M NaCl–0·01 N HCl. The fractions were identified and quantified by means of their ultraviolet absorption spectra.[15]

RESULTS AND DISCUSSION

In Table 1 are summarized the results of analyses of liver homogenate extracts from control and shocked rats, medicated and unmedicated, at the time the occluded bowel segment was released, and 2 hr thereafter. After 4 hr of occlusion the ATP concentration in the liver homogenate had declined to one-eighth of the control value, and remained depressed for at least 2 hr. Animals which received neomycin prior to occlusion had ATP concentrations which were consistently higher than those observed in the unmedicated groups.

The ADP analysis was complicated by the fact that even though the optical density at 260 mμ remained about the same, the absorption maximum had shifted to 250 mμ. To determine the cause of this shift the elution volume was increased. It then became apparent (Fig. 1) that this component was composed of three separate peaks.

No AMP was detected after 4 hr of occlusion, however it was detected 2 hr following release of occlusion. A peak with an absorption maximum of 250 mμ, eluted at the same point as AMP, was shown to be hypoxanthine (detected by paper chromatography).

Kleinzeller,[16] Krebs and Hems,[17] and Green *et al.*[18] have shown that the enzymic breakdown of ATP in muscle eventually leads to the formation of IMP. Webster,[19] working with washed myofibrils, detected a direct deamination of ADP to IDP. Although unable to detect IMP in the liver extracts chromatographed, two peaks

* The trade name of The Upjohn Company for neomycin sulfate is Mycifradin.

TABLE 1. ADENINE NUCLEOTIDE CONCENTRATIONS IN RAT LIVER HOMOGENATE

Group*	No. of animals	AMP (μmoles/g)	ADP (μmoles/g)	ATP† (μmoles/g)
1	10	0·78 ± 0·21‡	1·19 ± 0·17	2·54 ± 0·42
2	10	1·12 ± 0·24	1·47 ± 0·22	3·17 ± 0·56
3	10		1·36 ± 0·18	0·31 ± 0·14
4	10	0·85 ± 0·16	1·16 ± 0·21	1·86 ± 0·26
5	8	0·36 ± 0·17	0·48 ± 0·15	0·38 ± 0·24
6	9	0·84 ± 0·12	0·82 ± 0·11	1·09 ± 0·30

* Group 1: unmedicated control; group 2: medicated (neomycin) control; group 3: unmedicated, obstructed bowel, sacrificed at time of occlusion release; group 4: medicated, obstructed bowel, sacrificed at time of occlusion release; group 5: unmedicated, obstructed bowel, sacrificed 2 hr after occlusion release; group 6: medicated, obstructed bowel, sacrificed 2 hr after release of occlusion.

† In all instances, medicated vs. control, differences were significant at $P > 0{\cdot}01$.

‡ Standard deviation.

were found to have absorption spectra similar to those of the inosine nucleotides. The data presented agree with the findings of Hoffman *et al.*,[20] in which a substance from blood of patients in shock was detected which exhibited an absorption spectra similar to that of the inosine nucleotides. When the two peaks similar to IMP were chromatographed, using the solvent system of Webster,[19] one peak was found to be identical with IDP, whereas the other lay between IMP and IDP. Employing the solvent system of Krebs and Hems,[17] both peaks exhibited the same R_f as that of IMP. The two peaks similar to those of IMP may be either unstable cyclic isomers of IMP, similar

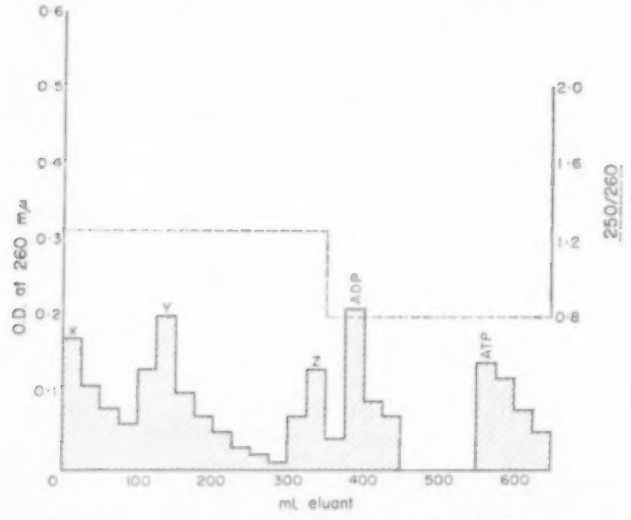


FIG. 1. Chromatographic separation of adenine and inosine nucleotides in homogenate of liver from unmedicated bowel-shocked animals, at time of occlusion release. Peak *X* was shown to be hypoxanthine; peaks *Y* and *Z* were chromatographically similar to IMP. Initial 350 ml of eluant, 0·003 N HCl; next 100 ml, 0·01 N HCl and 0·02 M NaCl; final 200 ml, 0·01 N HCl and 0·05 M NaCl.

to those reported for adenosine-2- and adenosine-3-phosphoric acids[21] with a ring from the 5 to 3, or 5 to 2 position, or substances readily degraded to IMP.

Extracts of liver from animals medicated with neomycin and subjected to bowel shock did not exhibit the hypoxanthine or IMP-like peaks, but instead maintained characteristic nucleotide peaks.

Rosenbaum *et al.*[13] have shown that a marked increase in the rate of phosphorylation occurs in the liver during hemorrhagic shock, leading to a depletion of the high-energy phosphate pool. Under conditions in which aerobic metabolism is sub-optimal, re-phosphorylation of ADP to ATP is depressed,[22] and degradation takes place.

The role of the inosine nucleotides detected in liver homogenates prepared from rats subjected to the bowel strangulation procedure remains unresolved.

SUMMARY

Adenine nucleotides have been quantitatively determined in homogenates of liver from control and bowel-shocked rats, with and without neomycin premedication. The ATP concentration was decreased to approximately one-eighth that of the control value in shocked animals; 2 hr after release of the occluded segment ATP remained depressed.

Homogenates of livers from unshocked animals which had received neomycin had significantly higher ATP concentrations than did unmedicated controls. Likewise, shocked animals which had received neomycin had ATP concentrations consistently higher than those of unmedicated rats.

Three unusual peaks were detected by chromatographic procedures in homogenates of liver from unmedicated, bowel-shocked animals. One peak was shown to be hypoxanthine, the other two were chromatographically similar to IMP.

Acknowledgements—We wish to thank Dr. William Haddon, Jr. for technical assistance, and Lucille P. Wendel for her stimulating discussions.

REFERENCES

1. J. F. CIER and M. TANCHE, *C.R. Soc. Biol., Paris* **148**, 1834 (1954).
2. L. THOMAS, *Ann. Rev. Physiol.* **16**, 467 (1954).
3. O. W. WENDEL, P. E. GRIFFIN and E. M. BROPHY, *Brit. J. Exp. Path.* **39**, 411 (1958).
4. E. C. GJESSING, *J. Biol. Chem.* **170**, 557 (1947).
5. O. W. WENDEL, E. M. BROPHY and J. J. AZUKAS, *Antibiot. Chemother.* **8**, 637 (1958).
6. J. WALKER, *Surgery* **19**, 825 (1946).
7. O. W. WENDEL and L. W. CHARKEY, *Proc. Soc. Exp. Biol. Med., N.Y.* **98**, 463 (1958).
8. G. A. LEPAGE, *Amer. J. Physiol* **146**, 267 (1946).
9. E. J. POTH, *Western J. Surg.* **60**, 205 (1952).
10. I. COHN, A. GELB and H. R. HAWTHORNE, *Ann. Surg.* **138**, 747 (1953).
11. S. J. SARNOFF and J. FINE, *Ann. Surg.* **121**, 74 (1945).
12. F. SCHWEINBURG, E. FRANK, A. SEGEL and J. FINE, *Proc. Soc. Exp. Biol., N.Y.* **66**, 45 (1947).
13. D. K. ROSENBAUM, E. D. FRANK, A. M. RUTENBURG and H. A. FRANK, *Amer. J. Physiol.* **188**, 86 (1957).
14. W. E. COHN and C. E. CARTER, *J. Amer. Chem. Soc.* **72**, 4273 (1950).
15. H. M. KALCKAR, *J. Biol. Chem.* **167**, 445 (1947).
16. A. KLEINZELLER, *Biochem. J.* **36**, 729.
17. H. A. KREBS and R. HEMS, *Biochim. et Biophys. Acta* **12**, 172.
18. I. GREEN, J. R. C. BROWN and W. F. H. M. MOMMAERTS, *J. Biol. Chem.* **205**, 493 (1953).
19. H. L. WEBSTER, *Nature, Lond.* **172**, 453 (1953).
20. G. T. HOFFMAN, A. ROTTINO and H. ALBAUM, *Science* **144**, 188 (1951).
21. D. M. BROWN, D. I. MAGRATH and A. R. TODD, *J. Chem. Soc.* 2708 (1952).
22. F. E. SAMSON, W. M. BALFOUR and N. A. DAHL, *Amer. J. Physiol.* **196**, 325 (1959).

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 59–64. Pergamon Press Ltd., Printed in Great Britain.

THE EFFECT OF CARBON TETRACHLORIDE, ETHIONINE AND CHLORETONE ON THE ASCORBIC ACID CONTENT OF RAT LIVER

MARY L. C. BERNHEIM

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina

(*Received* 23 *November* 1960)

Abstract—In rats with acute CCl_4 poisoning, the ascorbic acid content of the liver is very low. Chloretone (chlorobutanol) given to normal rats much increases the ascorbic acid content of the liver, but when given to rats poisoned with CCl_4, it does not. In rats which have received injections of ethionine, the ascorbic acid of the liver is within the normal range. Chloretone, given to such rats, lowers the ascorbic acid value of the liver. This apparent reversal of the action of Chloretone, which occurs when the synthetic mechanism for ascorbic acid is not disturbed, is at present not explained.

INTRODUCTION

THERE are several studies of the effect of CCl_4 on the vitamin content of the liver. Shils *et al.*[1] found a decrease of vitamin A and of certain of the vitamin B group in the livers of CCl_4-poisoned rats. Using liver slices and mitochondrial preparations, Christie and Judah[2] concluded that CCl_4 damages the mitochondria. Vitamin E has also been studied in relation to CCl_4 poisoning.[3] Ascorbic acid, however, has not been studied, possibly because it is not a vitamin for the rat.

Homogenates of most normal tissues form fatty acid peroxides during incubation and these react with 2-thiobarbituric acid to give a red color. Ascorbic acid is necessary for the oxidation,[5] and the amount of color formed in homogenates can usually be increased by the addition of ascorbic acid to the incubating mixture. It was noted that the color formed in liver homogenates of rats with acute CCl_4 poisoning was much reduced, and that the addition of ascorbic acid to the mixture before incubation resulted in a marked increase of color. This suggested a low ascorbic acid content in the poisoned tissues. Therefore, the ascorbic acid concentration in rats livers after CCl_4 administration was determined and compared to that of the livers of rats given ethionine. The effect of Chloretone (chlorobutanol), which is known to stimulate the synthesis of ascorbic acid, was also investigated.

EXPERIMENTAL

Five groups of rats were used. Groups 1, 2 and 3 consisted of a mixed lot of Wistar rats of different ages and sexes. The animals of group 1 were injected intraperitoneally with a single dose of CCl_4, 1 mg/g or sometimes less; they were killed by a blow on the head, usually 17 hr later. Those of group 2 were injected with Chloretone, 30 mg/rat, dissolved in 0·1 ml ethyl alcohol, and the rats were killed at 17 hr. The animals

of group 3 received both substances and were similarly treated. Groups 4 and 5 consisted of female rats which were fasted for 12 hr and then were given ethionine, 75 mg/100 g, divided into three doses at intervals of 2½ hr as described by Koch-Weser *et al.*;[6] they were killed 48 hr later. The animals of group 5 were injected with Chloretone 17 hr before death.

The livers were removed and iced, and 1 g was weighed and homogenized with 9 ml of water, or, for ascorbic acid determinations, with 5% metaphosphoric acid.

The ascorbic acid in the liver was determined by the method of Roe[7]. The metaphosphoric acid homogenate was centrifuged and 1 ml of the supernatant fraction, which was clear or slightly opalescent, was treated with 9 ml of a solution of 2:6-dichlorophenolindophenol in sodium acetate solution and the color was read at once in the Evelyn colorimeter at 520 mμ. The dye was then decolorized completely by the addition of excess ascorbic acid and the value given by the blank was subtracted from the experimental values. The amount of ascorbic acid corresponding to the amount of reduced dye was read from a standard curve.

In order to estimate the total ascorbic acid (the reduced and the dehydroform), the method described by Roe[8] was used. Ascorbic acid was oxidized by treatment with activated Norit, and the color formed with dinitrophenylhydrazine-thiourea and sulfuric acid was estimated in the Evelyn colorimeter at 540 mμ. For this determination the liver homogenate was diluted 1 : 25 with 6% trichloracetic acid, centrifuged and Norit was added. After filtration, 4 ml were used for the determination.

The total nitrogen values were determined on the liver water homogenates, using 2 ml of a 1 : 1000 dilution. The usual Kjeldahl procedure was employed, followed by Nesslerization, and the color was read at 420 mμ. For dry weights, a 3 ml sample of the 1 : 10 water homogenate was dried and weighed.

TABLE 1. THE EFFECT OF CCl_4 ON THE ASCORBIC ACID CONCENTRATION IN RAT LIVER

	Dry weights g/100 g liver	Nitrogen g/100 g liver	Ascorbic acid mg/100 g liver	mg ascorbic acid / g nitrogen
Normal male rat	27·6	2·7	23·0	8·5
Normal female rat	30·7	3·5	27·0	7·8
Male rats after CCl_4	27·4	3·9	11·5	2·9
	26·9	3·3	14·0	4·2
Female rats after CCl_4	20·6	2·15	7·8	3·6
	20·0	2·15	7·0	3·3

RESULTS

Table 1 shows the results of ascorbic acid determinations on the livers of rats with acute CCl_4 intoxication. A definite reduction was found in the amount of ascorbic acid in these livers.

Under the conditions of these experiments, the livers of female rats treated with CCl_4 were grossly larger and appeared more fatty than the livers of males. This impression was confirmed by the dry weights and total nitrogen content of the livers, as can be seen in Table 1. In the table, the data for two individual female and two male

rats are given, and experimental values were obtained for many others. For instance, in a group of five normal rats (three males and two females) the mean total nitrogen content of the livers was 3·2 g per 100 g wet weight, and ascorbic acid value was 23·6 mg, with a ratio of mg of ascorbic acid/g of total N of 7·4. The corresponding figures for six male rats treated with CCl_4 were, nitrogen, 3·3 g, ascorbic acid 14·1 mg, and a ratio of 4·4. For a group of eight similarly treated females, the nitrogen was 2·15 g, the ascorbic acid 7·8 mg, and the ratio 3·6. It can be seen that the ascorbic acid content of the CCl_4-treated animals is low, expressed either in terms of wet weight or nitrogen content. This is true for both males and females.

Dehydroascorbic acid is not estimated under the conditions employed for ascorbic acid, so it was possible that, in CCl_4-poisoned livers, the low values for ascorbic acid might be the result of a change of equilibrium between the two forms, and the oxidation of a greater part than normal, without change in the total amount. In order to test this, the total ascorbic acid was estimated after the oxidation of the ascorbic acid fraction with activated Norit. The values for two normal rats were, ascorbic acid, 18·6 and 22·0 and the total ascorbic acid 20·0 and 25·5 mg/100 g of liver, and for two rats which had been injected with CCl_4 the values were 4·0 and 3·7, and 4·2 and 4·0, respectively. Therefore, the low amounts of ascorbic acid found in the CCl_4-livers are not the result of oxidation of part of the acid, but represent a real lowering of the total amount present.

The reduction of the ascorbic acid content of the livers of rats treated with CCl_4 is rapid. A few experiments showed that the process is already beginning after 5 hr, and is maximal at from 12 to 15 hr after the CCl_4 injection. Recovery follows relatively slowly, and at 48 hr the ascorbic acid values were still lower than normal.

It seemed possible that fasting might contribute to changes in the concentration of ascorbic acid in the liver. Animals poisoned with CCl_4 eat little if any food, so some experiments were carried out to determine the ascorbic acid values on fasting rats. Food was removed for 15 hr during the night before they were killed. The values for ascorbic acid were as high as or higher than normal, and a group of five rats averaged 27·4 mg/100 g.

A remarkable list of drugs, chemically unrelated to one another, cause an increase in the urinary output of ascorbic acid in rats and other animals.[9] Among these Chloretone is especially effective and increases the excretion in rats to amounts up to 100 times the normal range. Therefore, the effect of this drug on the ascorbic acid content of CCl_4 livers was investigated.

Single doses of Chloretone (30 mg) were given by intraperitoneal injection to rats, which were killed 17 hr later. Table 2 shows that this drug increased the ascorbic acid content of the normal liver, a finding which was to be expected in view of its known effect on the urinary excretion of the acid. However, in rats receiving Chloretone with CCl_4 (1 mg/g) the stimulation of the production of ascorbic acid was prevented, and the values resembled those obtained after CCl_4 alone; smaller doses of CCl_4 caused this effect. For instance, in a series of twenty rats, four normal ones gave an average value for the ascorbic acid of the liver of 23·0 mg/100 g. Six rats were given 30 mg of Chloretone alone and the mean value found was 36·6 mg. For four rats which received 30 mg of Chloretone, and 1·0 mg of CCl_4 per g, the value was 7·5 mg; four receiving 0·5 mg of CCl_4 per g gave 10·4 mg/100 g, and two receiving 0·25 mg of CCl_4 per g gave 8·4 mg of ascorbic acid per 100 g. Even increasing the dose of

Chloretone up to 100 mg in three rats did not increase the low ascorbic acid values caused by 0·25 mg of CCl_4 per g.

For comparison, the ascorbic acid content of fatty livers produced by ethionine was determined, using female rats only. Forty hours after the injection, when maximum damage should have occurred,[6] the animals were killed and the livers homogenized. The mean value for the ascorbic acid for four rats was 18·4 mg/100 g of liver (compared to 23·6 for untreated rats and 7·8 for female rats after CCl_4), while the ratio

TABLE 2. THE EFFECT OF CHLORETONE ON THE ASCORBIC ACID CONTENT OF THE LIVER OF RATS TREATED WITH ETHIONINE OR CCl_4

	Nitrogen g/100 g liver	Ascorbic acid mg/100 g liver	mg ascorbic acid / g nitrogen
Chloretone	3·3	36·0	10·9
	3·1	32·5	10·5
	3·1	28·5	9·1
CCl_4	2·15	7·8	3·6
	2·15	7·0	3·3
	3·3	14·0	4·2
CCl_4 and Chloretone	3·1	17·0	5·5
	3·1	14·0	4·5
	3·1	10·5	3·4
Ethionine	2·67	19·3	7·2
	2·70	23·5	8·7
	2·36	15·7	6·6
	2·56	15·0	5·8
Ethionine and Chloretone	2·90	9·5	3·3
	2·67	12·0	4·5
	1·95	12·5	6·4
	2·59	10·0	3·9

ascorbic acid : nitrogen remained within the normal range (Table 2). The effect of ethionine is thus in marked contrast with that of CCl_4.

Chloretone was given to rats which had received ethionine 24 hr previously. The animals were killed 17 hr after the Chloretone injection. The results show that Chloretone, in contrast to its effect on normal or CCl_4 livers, definitely lowers the ascorbic acid content of the livers of the ethionine-treated animals (Table 2).

DISCUSSION

Christie and Judah[2] were able to show that the changes in the vitamin B complex reported by Shils *et al.*[1] in CCl_4-treated rats were related to the leakage of pyridine nucleotides from the mitochondria, and consequent disorganization of the enzymes of the Krebs cycle. The activity of these enzymes could be completely or partially restored by the addition of the coenzymes *in vitro*. Attempts have been made to protect animals by the addition of vitamins to the diet. Hove and Hardin[3], after finding evidence that vitamin E was deficient in CCl_4-poisoned animals, were able to increase the survival rate of rats receiving one intraperitoneal dose of CCl_4 by injecting them with vitamins E and B_{12}. Nowhere, however, is there reference to vitamin C and the

Vol. 7
1961

effect of injections of CCl_4 on the concentration of the vitamin in the liver, nor of attempts to alleviate the damage by giving it.

The results shown in this paper indicate that there is a rapid lowering of the vitamin C concentration in the CCl_4-poisoned liver of rats. A few hours after a single intraperitoneal injection of CCl_4 the concentration of ascorbic acid in the liver is reduced; the reduction is maximal between 12 and 17 hr; the values then begin to rise again, but recovery is not complete in 48 hr.

These results are in contrast to those obtained when ascorbic acid was measured in the livers of ethionine-treated rats. In these fatty livers, the vitamin C values per 100 g wet weight were slightly lower than normal, but the ratio of ascorbic acid per gram of nitrogen remained within normal limits. The fatty infiltration without necrosis, typical of ethionine poisoning, does not affect the synthesis of vitamin C by the liver.

Ethionine has been shown to be more toxic for the livers of female rats than for those of males.[10] There is a tendency for the same sex difference under the conditions of these experiments with CCl_4, but the effect is not so marked.

Of the many drugs which increase the urinary output of vitamin C, Chloretone was chosen because it increases the daily vitamin C content of rat urine, up to 100 times the normal range. In guinea pigs, which cannot synthesize vitamin C, the D-glucuronic acid content of the urine rises after barbital,[11] suggesting that there is an increased synthesis of substances which, in most animals, yield ascorbic acid. There is no obvious reason for the increased ascorbic acid formation. Increased formation of glucuronic acid from glucose does not have any known relationship to the metabolism of the group of drugs, which, indeed, have no common chemical structure. Some of them are metabolized, but some are excreted unchanged. Moreover, an increased utilization of glucuronic acid, such as occurs when α-naphthol or borneol are conjugated, does not alter the output of vitamin C. It has been suggested that the effect of the drugs is a hormonal one, for it does not occur in hypophysectomized animals.

In these experiments, Chloretone increased the concentration of vitamin C in the livers of normal rats. However, when given to animals which had received CCl_4, no increase was observed. Even one-quarter the dose usually given was enough to prevent the Chloretone effect.

The effect of Chloretone given to ethionine-treated rats is quite different. In these livers, in which, judging from their ascorbic acid content, the mechanism for the production of ascorbic acid is still intact, Chloretone causes a reduction, rather than an increase, in the amount present. Koch-Weser *et al.*[6] discuss in detail the differences between the lesions of CCl_4- and ethionine-treated animals. Their description shows clearly that the fatty accumulations following the injection of ethionine are not associated with necrosis, nor did they find changes in the activity of certain enzymes. After CCl_4, however, necrotic and regenerative changes were also present, and enzyme activities were altered. These include increased alkaline phosphatase and decreased esterase activity. However, the experiments reported in this paper show that, even in the ethionine-fatty liver, the process by which ascorbic acid is synthesized is not normal. This is also indicated in a recent paper[12] which shows that barbital, which, like Chloretone, increases the excretion of ascorbic acid, does not do so in ethionine-treated rats. The mechanism of the changes cannot be explained.

REFERENCES

1. M. E. SHILS, M. SASS, M. WOLKE, G. MARKS, L. J. GOLDWATER and A. BERG, *Brit. J. Ind. Med.* **8**, 284 (1951).
2. G. S. CHRISTIE and J. D. JUDAH, *Proc. Roy. Soc.* B **142**, 241 (1954).
3. E. L. HOVE and J. O. HARDIN, *Proc. Soc. Exp. Biol., N.Y.* **77**, 502 (1951).
4. F. BERNHEIM, M. L. C. BERNHEIM and K. M. WILBUR, *J. Biol. Chem.* **174**, 257 (1948).
5. A. OTTOLENGHI, *Arch. Biochem. Biophys.* **79**, 355 (1959).
6. D. KOCH-WESER, E. FARBER and H. POPPER, *A.M.A. Arch. Path.* **51**, 498 (1951).
7. J. H. ROE, *Meth. Biochem. Anal.* **1**, 121 (1954).
8. J. H. ROE, *Meth. Biochem. Anal.* **1**, 127 (1954).
9. H. E. LONGENECKER, H. H. FRICKE and C. G. KING, *J. Biol. Chem.* **135**, 497 (1940).
10. E. FARBER, M. V. SIMPSON and H. TARVER, *J. Biol. Chem.* **182**, 91 (1950).
11. J. J. BURNS, C. EVANS and N. TROUSOF, *J. Biol. Chem.* **227**, 785 (1957).
12. O. TOUSTER, R. W. HESTER and R. A. SILER, *Biochem. Biophys. Res. Com.* **3**, 248 (1960).

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 65–74. Pergamon Press Ltd., Printed in Great Britain.

METABOLISM OF BIS-β-CHLOROETHYL SULFIDE (SULFUR MUSTARD GAS)*

CLARKE DAVISON, ROBERT S. ROZMAN† and PAUL K. SMITH‡

Department of Pharmacology,
The George Washington University School of Medicine, Washington, D.C.

(*Received* 8 *December* 1960)

Abstract—The metabolism of bis-β-chloroethyl sulfide-^{35}S has been investigated in mice, rats and human subjects. In the rodents, the major portion of radioactivity was excreted in the urine within the first 24 hr, whereas in the human considerable radiosulfur was retained for long periods. In the rat only traces of radioactivity were expired, while small amounts were found in the feces. Large amounts of thiodiglycol and of bis-β-chloroethyl sulfone have been detected in the urine, largely as conjugates. Evidence is presented that much of the drug reacts immediately with glutathione, and this complex is excreted. It is concluded that the majority of the radioactivity excreted in the urine represents compounds formed from alkylation by the drug, rather than metabolites formed by enzymic action.

INTRODUCTION

Bis-β-chloroethyl sulfide (mustard gas, Yperite) represents the oldest of the so-called alkylating agents, having been synthesized first in 1822. It received usage as a vesicant during the first World War. Its clinical employment as a tumor inhibitor has been minimal, probably resulting from the inconvenience of handling the volatile, water-insoluble drug, rather than from any lack of effectiveness, since reports[1, 2] suggest that it possesses tumor-inhibiting activity similar to that of the various other "alkylating" compounds.

While a variety of investigators have studied *in vitro* reactions of bis-β-chloroethyl sulfide with various amino acids,[3, 4] proteins[5] and nucleic acid constituents,[6, 7] the degradation of this compound within the mammalian body has been largely neglected.

The present studies of its metabolism were undertaken not only because of an interest in the metabolism of alkylating agents, but also in the belief that such information might give some insight into the mechanism of action of this general class of compounds. It is believed because of its simplicity of structure that bis-β-chloroethyl sulfide represents the ideal compound for such studies.

MATERIALS AND METHODS

Synthesis of sulfur-labeled bis-β-chloroethyl sulfide and possible metabolites

Radioactive bis-β-chloroethyl sulfide was synthesized according to modifications of the method of Boursnell *et al.*[8] by reaction of hydrogen sulfide-^{35}S with stoichiometric amounts of ethylene oxide to yield thiodiglycol, followed by chlorination with

* This research was supported in part by Research Grant CY-308(C10) from the National Cancer Institute, United States Public Health Service, and by a grant from the American Cancer Society.

† Presented to the Columbian College of The George Washington University in partial fulfillment of the requirements for the degree of Master of Science.

‡ Deceased October 1960.

concentrated hydrochloric acid. The technique differed from that of Boursnell *et al.* in that the hydrogen sulfide generated from barium sulfide-^{35}S was permitted to diffuse into the reaction vessel in a closed system rather than being flushed over with hydrogen, losses being minimized in this way. Usually about 1 m-mole of barium sulfide, containing 10 mc of radioactive sulfide (from the Oak Ridge National Laboratory), was used. The bis-β-chloroethyl sulfide formed was extracted from the acidic solution with low-boiling petroleum ether and, after removal of the organic solvent, was sublimed *in vacuo*. Yields ranged up to 91 per cent. The radioactive product was frozen in sealed ampules until used. The purity of the product was established by melting point, and by ascending chromatography on Whatman no. 1 paper in two non-aqueous systems, chloroform on formamide-saturated paper (R_f, 0·78), and petroleum ether on silicone-impregnated paper (R_f, 0·75). To prevent volatilization of the compounds during counting, the chromatograms were sprayed with collodion-ether.

Radioactive thiodiglycol was prepared by shaking the bis-β-chloroethyl sulfide-^{35}S in 0·1 N sodium hydroxide for 1 hr. Its purity was established by ion exchange chromatography on Dowex-1, as described below.

A number of possible metabolites of bis-β-chloroethyl sulfide were synthesized: sulfoacetic acid;[9] potassium isethionate;[10] bis-β-chloroethyl sulfoxide and sulfone;[11] and thiodiglycolic acid-sulfone.[12]

PROCEDURE FOR METABOLISM STUDIES

Rats

Twenty-two male Wistar rats weighing from 150 to 300 g were injected intravenously with radioactive bis-β-chloroethyl sulfide in one of four solvents. The solvents, propylene glycol, 95% ethanol, dioxane or 20% ethanol–80% Lipomul*, did not cause appreciable hydrolysis of the drug during the period of administration. The dosages employed were from 1 to 5 mg of drug per kg in 1 ml of solvent per kg, solutions being used within 1 min of preparation. The solvents themselves did not produce grossly evident toxicity, except for a brief hematuria that occurred in certain of the animals given propylene glycol. Toxicity resulting from the drug during the period of observation of up to 72 hr consisted of diarrhea, emaciation, convulsions and sloughing at the site of injection. Since toxicity seemed least in the ethanol–Lipomul solvent this solvent was employed in all the later experiments. Urine collections were made by restraining the animals in a rigid frame suspended over the collecting tube. Collections were made during the periods of 0–6, 6–12, 12–24, 24–48 and 48–72 hr and rat urine and feces were analyzed by chromatographic and chemical techniques. In one animal the expired air was passed through successive traps of lead acetate, potassium permanganate, and Pirie's reagent[13] in an effort to collect volatile sulfur compounds, such as sulfides, mercaptans and sulfones.

Mice

Twenty male CAF_1 mice were injected intravenously with radioactive bis-β-chloroethyl sulfide in propylene glycol at a dose of 2 mg/kg. Toxic reactions were observed as with rats. Urine was collected in a metabolism cage at 0–6, 6–12, 12–24 and 24–48 hr and the radioactivity of the urine fractions was measured.

* Lipomul is an emulsion containing 15% (w/v) vegetable oil and 4% anhydrous dextrose, manufactured by the Upjohn Company, Kalamazoo, Mich.

Vol. 7
1961

Human subjects

Two human terminal cancer patients were injected intravenously with 5 mg of radioactive bis-β-chloroethyl sulfide dissolved in 5 ml of 95% ethanol. The solution was injected over a 10-min period into the tubing of an infusion apparatus during the administration of isotonic saline.* Total urine output was collected at 6, 12, 24 and 48 hr; aliquots were subjected to chromatography in the usual manner and their radioactivity was measured. Blood samples were taken 1, 3, 5, 15 and 60 min and 6, 12, 24 and 48 hr after termination of the administration. The plasma and cells were checked for total radioactivity.

Separation of urinary metabolites

Two ion exchange resins, Dowex 50-X8, 200–400-mesh, and Dowex 1-X8, 200–400-mesh, were employed for chromatography of the radioactive urine. Dowex-50 cation exchange columns (1 cm × 50 cm) were washed with 4 N NH_4OH, then equilibrated with 0·1 HCl and suitable aliquots of urine were eluted successively with 0·1 N HCl, pH 1; 0·1 M citric acid, pH 5·5; and 4 N NaOH. Volumes of 10–20 ml were collected and suitable aliquots were counted.

Dowex-1 anion exchange columns (1 cm × 50 cm) were washed with 4 N HCl and were equilibrated with 0·1 N NH_4OH, and urine samples were eluted successively with 0·1 N NH_4OH, pH 11; 0·1 N ammonium acetate, buffered to pH 5 with acetic acid; 0·1 N acetic acid, pH 2·8; 0·1 N formic acid, pH 2·3; 0·1 N HCl, pH 1 and finally 4 N HCl (pH about 0).

RESULTS

Blood levels in the human

The plasma and red cells showed only slight radioactivity. Calculation revealed that after several minutes from 80 to 90 per cent of the radioactivity disappeared from the blood. The residual level of radioactivity remained constant in both plasma and cells for at least 2 days, indicating a relatively permanent binding to some blood constituent, possibly protein. Other workers have also found that the alkylating agents disappear very rapidly from blood.[14]

Excretion of radioactivity

Table 1 presents the percentage of radioactivity from bis-β-chloroethyl sulfide excreted over various intervals in the three species studied. The trace of activity found in expired air was too little to permit any chemical identification. It is apparent from Table 1 that the major portion of the drug emerges rather rapidly from the rodent body, either due to an excessive amount over the available reaction sites, or due to the formation of conjugates with small, rapidly-excreted molecules, or possibly by the excretion of large molecules from damaged renal tubules. In the human subject, in which the dosage was of the order of 0·1 mg/kg, rather than the higher amounts (1–5 mg/kg) given the small animals, the urinary excretion of radioactivity was relatively poor over the 48-hr period. This may reflect the lower overall rate of metabolism of the human, or the fact that at lower dosages the molecules reacting first are those more slowly turned over, i.e. proteins, nucleoproteins, etc. Moreover, the patients were in terminal stages of cancer and may well have had pre-existing metabolic disorders, especially anuria and ascites.

* The co-operation of Dr. Louis Alpert of The George Washington University Hospital is greatly appreciated.

Boursnell *et al.*[15] have reported excretion of radioactivity in bile after administration of bis-β-chloroethyl sulfide-^{35}S. Bile cannulation was performed in two rats, and the bile collected was analyzed by paper chromatography. Radioactivity was detected in neither taurine nor taurocholate.

TABLE 1. EXCRETION OF RADIOACTIVITY AFTER INTRAVENOUS ADMINISTRATION OF BIS-β-CHLOROETHYL SULFIDE-^{35}S

Route of excretion	Period after drug (hr)	Per cent of injected radioactivity		
		Rat	Mouse	Man
Expired air	0–6	0·05	—	—
Feces	0–12	6	—	—
Urine	0–6	48	48	6·4
	6–12	25	22	10·6
	12–24	5	11	5·5
	24–48	9	5	4·0
	48–72	2	4	—

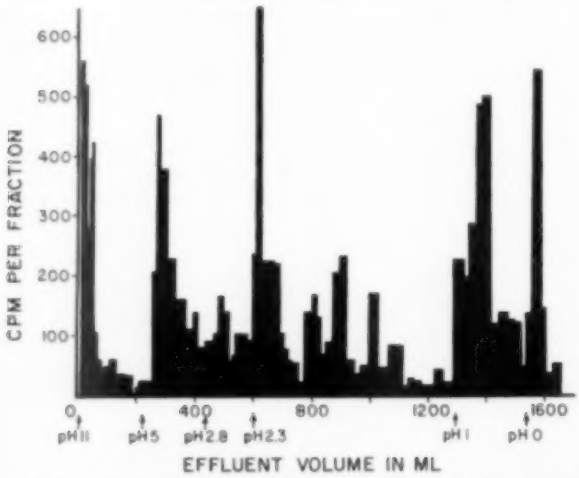


FIG. 1. Eluant pattern from Dowex-1 of sulfur-35 excretion after administration of bis-β-chloroethyl sulfide-^{35}S. Forty-eight-hour sample of human urine.

Chromatographic studies with urine

Preliminary chromatography of rat and human urine on Dowex-50 revealed that 98 per cent of the radioactivity emerged immediately from the column at pH 1; most of the remainder emerged at pH 5·5, a finding which implied that most of the metabolites were present in a neutral or anionic form.

Chromatography on Dowex-1 revealed an excretory pattern characteristic of a host of metabolites. Fig. 1 shows the elution pattern derived from a sample of human urine. The patterns derived from rat and mouse urine were essentially similar to each other but differed from those of the human subjects in showing somewhat lower peaks at pH 2·8 and 2·3. Fig. 2 portrays a representative diagram of radioactivity in rat urine after the administration of either bis-β-chloroethyl sulfide-^{35}S or of an identical

Vol. 7
1961

dose of its hydrolysis product thiodiglycol-^{35}S. It appears that some but not all of the metabolites may be derived from the latter rather than from the sulfur mustard.

Indentification of urinary metabolites in the rat

Chromatography on Dowex-1 had revealed the presence, in appreciable amounts, of at least six substances in rat urine. The major radioactive compounds were a neutral or very weakly acidic substance emerging from the column at pH 11 and a more anionic substance emerging at pH 5. A number of likely sulfur compounds were subjected to the same chromatographic scheme in order to determine their elution characteristics.

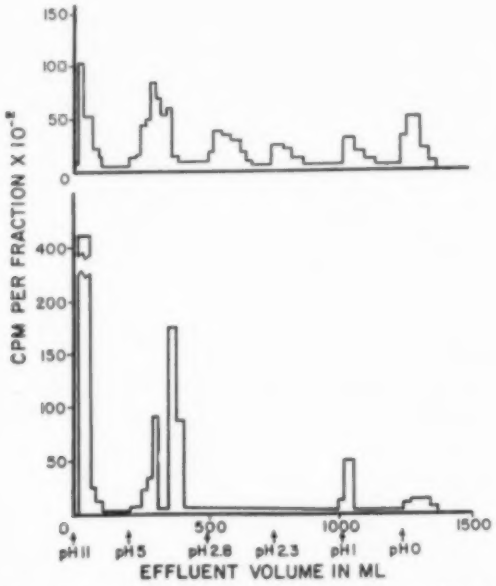


FIG. 2. Eluant pattern from Dowex-1 of sulfur-35 excretion in 6-hr samples of rat urine. Upper: after administration of bis-β-chloroethyl sulfide-^{35}S. Lower: after administration of bis-β-hydroxyethyl sulfide-^{35}S.

Identification of thiodiglycol

Since thiodiglycol emerged immediately from the Dowex-1 column at pH 11, the corresponding major component of urine was analyzed for thiodiglycol by ascending paper chromatography on Whatman no. 1. In four solvent systems: (1) phenol saturated with water (R_f, 0·88); (2) isoamyl alcohol saturated with water (R_f, 0·69); (3) 95% ethanol–NH_4OH 95 : 5 (v/v) (R_f, 0·82); (4) 95% ethanol-concentrated HCl 95 : 5 (v/v) (R_f, 0·88); a portion of the radioactivity present in this fraction had the same R_f as thiodiglycol.

Quantitation of the amount of thiodiglycol present was effected by inverse isotope dilution. To suitable samples of the whole urine was added 0·2 m-moles of carrier thiodiglycol, and the di-*p*-nitrobenzoyl ester was prepared according to McElvain[16]. The ester was identified by melting point and mixed melting point, and was repeatedly recrystallized from absolute ethanol until constant specific activity was reached. In repeated determinations free thiodiglycol accounted for about 6 per cent of the first

fraction or 1·4 per cent of the total radioactivity. A similar value was obtained upon preparation of the di-α-naphthyl carbamate of thiodiglycol.

To detect conjugates of thiodiglycol in urine, whole urine was hydrolyzed by refluxing with dilute hydrochloric acid for 2 hr, after carrier thiodiglycol had been added. The di-α-naphthyl carbamate ester was synthesized and purified from ethanol as above. Thiodiglycol accounted for up to 14·4 per cent of the total radioactive metabolites, a tenfold increase over the free form. Hydrolyses in base and by β-glucuronidase gave 3·0 and 2·6 per cent, respectively, findings which suggest conjugation with glucuronic acid, as well as with other compounds.

Identification of bis-β-chloroethyl sulfoxide

Partial oxidation of the sulfur of bis-β-chloroethyl sulfide produces the sulfoxide. Carrier sulfoxide added to the urine and recrystallized to constant specific activity, as well as carrier sulfoxide added with the isolation of the phenyl mercaptan derivative,[11] showed that up to 0·5 per cent of the activity of whole urine can be accounted for as the sulfoxide.

Identification of bis-β-chloroethyl sulfone or derivatives

Further oxidation of the sulfoxide would yield the sulfone. Carrier sulfone isolated directly from radioactive urine contained 1·0 per cent of the total radioactivity. The urine was then examined for the compound by dehydration to the thioxane, according to a modification of the procedure of Cashmore[17]. Whole urine was refluxed for 6 hr with carrier sulfone in 1·7 N NaOH. The solution was then concentrated and extracted with chloroform. The chloroform was evaporated and the residue recrystallized to constant activity from ethanol. The thioxane formed accounted for 7·4 per cent of the total radioactivity of the rat urine, indicating that the major portion of the sulfone is present as conjugates. A similar experiment in which the thioxane itself (rather than sulfone) was added directly and isolated, revealed that no radioactivity was present as this compound, suggesting that this compound is not normally formed.

Identification of isethionic acid

This substance results from oxidation and splitting of the sulfide with simple hydrolysis of the halogen atoms. It emerged from the columns along with sulfoacetic acid. Whole urine, as well as this fraction, was analyzed for isethionate by inverse isotope dilution, isolating the potassium acetyl derivative.[10] This metabolite accounted for 0·2 per cent of the total.

Identification of inorganic sulfate

Complete oxidation of bis-β-chloroethyl sulfide leads to sulfate formation. This ion emerged, together with the sulfonic acids, from the columns in the pH 0 fraction. Whole urine was examined for sulfate by addition of carrier and recovery as benzidine sulfate after phosphate precipitation with magnesium carbonate–ammonium chloride.[18] Since benzidine sulfate does not recrystallize readily, purification was effected by repeatedly dissolving the precipitate in base, removing the insoluble benzidine, acidification of the supernatant fraction and reprecipitation with fresh benzidine. Again, only traces of activity were detected (0·5 per cent). No appreciable increase in radioactivity as sulfate was observed upon acid hydrolysis, a finding which indicates that no sulfur-35 was present as ethereal sulfate.

Vol. 7
1961

Identification of sulfoacetic acid

It was proposed that sulfoacetic acid might result from oxidation of the sulfur and terminal carbon of the molecule after splitting of one carbon–sulfur bond. This highly acidic compound emerged from Dowex-1 with 4 N HCl. Accordingly, that fraction and whole urine were tested for sulfoacetic acid by inverse isotope dilution, and isolation of the aniline salt of sulfoacetic acid–anilide according to McElvain[19]. The compound was identified by melting point and recrystallized repeatedly from 95 per cent ethanol. Only a trace (0·2 per cent) of sulfoacetate was detected. This represented about 10 per cent of the eluted fraction. No appreciable increase in recovery of radioactive sulfoacetate was observed upon acid hydrolysis.

Identification of thiodiglycolic acid

Thiodiglycolic acid, formed by oxidation of both β-carbons of the otherwise intact bis-β-chloroethyl sulfide molecule, was eluted from the Dowex-1 columns at pH 1. The acid was isolated from this fraction and from whole urine as the dianilide derivative after the addition of carrier. It accounted for only 0·2 per cent of the total urinary activity. Isolation of thiodiglycolate after acid hydrolysis accounted for 1·0 per cent, so that apparently 0·8 per cent is present as conjugates.

Identification of sulfone of thiodiglycolic acid

In view of the recovery of bis-β-chroloethyl sulfone and of small amounts of thiodiglycolic acid, it was suspected that the sulfone of thiodiglycolic acid might be detected. Carrier amounts of thiodiglycolic acid sulfone were added to urine which was then extracted repeatedly with ether. After evaporation of the ether the acid was repeatedly recrystallized from hot toluene by adding glacial acetic acid until the acid just dissolved, and then cooling the solution. The sulfone accounted for 4·8 per cent of the urinary activity.

Identification of the reaction products of bis-β-chloroethyl sulfide and its sulfone with cysteine

Since the comparatively simple organic compounds accounted for only one-quarter of the excretion of radioactivity after bis-β-chloroethyl sulfide administration, conjugates with cysteine were investigated. Cysteine was selected for study since it possesses the reactive sulfhydryl group and since its reaction products had already been reported by Hartwell[3]. These two derivatives were isolated as white solids, the formol titration values of which corresponded to a product of two molecules of cysteine reacting with one of bis-β-chloroethyl sulfide or its sulfone. Carrier amounts of the sulfide or sulfone conjugate were mixed with urine, dissolved by the dropwise addition of concentrated HCl and precipitated by the addition of ammonium hydroxide. Reprecipitations were from sodium hydroxide solutions by the addition of acid, and by acetone precipitation. Thirteen precipitations were performed until constant specific activity was reached. No radioactivity was detectable in the cysteine–sulfide conjugate, while a trace (0·2 per cent) was found in the conjugate with the sulfone.

Identification of the reaction products of bis-β-chloroethyl sulfide with glutathione

Since glutathione also has the reactive sulfhydryl group and is found in relatively high concentration in the plasma, the possibility of its interaction with bis-β-chloroethyl sulfide was studied. Several preparations were made with various ratios of glutathione to the drug, but in agreement with Hartwell[3] a gummy oil was repeatedly

obtained. The reaction products, upon paper chromatography in 60% ethanol saturated with potassium chloride or in pyridine–isoamyl alcohol–water (80 : 40 : 70 v/v), seemed to have appreciable amounts of glutathione remaining. The products were largely freed of glutathione by column chromatography on Dowex-1 anion exchange resin. Glutathione conjugates emerged at pH 5, whereas the glutathione itself was eluted with 0·1 N acetic acid. Preparations of the product were mixed with radioactive urine and subjected to such chromatography. The fractions obtained at pH 5 were lyophilized, taken up in minimal volumes of water and repeatedly reprecipitated with acetone or acetone–chloroform to constant specific activity. About 45 per cent of the total radioactivity was recovered in the oil after seven precipitations. Examination of the total urinary excretion curve reveals (Fig. 2) that the pH 5 peak could well contain such a large percentage. Because of the unknown composition of these reaction products, and their hygroscopic nature, the accuracy of this quantitative value must remain in question; both column and paper chromatography failed to separate the radioactivity from the reaction products.

TABLE 2. URINARY METABOLITES 6–12 HR AFTER ADMINISTRATION OF BIS-β-CHLOROETHYL SULFIDE-^{35}S TO RAT

Urinary fraction	Proposed metabolite	Per cent of total urinary radioactivity
Whole urine	Glutathione-bis-β-chloroethyl sulfide conjugates	45·0
Whole urine	Glutathione-bis-β-chloroethyl sulfone conjugates	7·0
Whole urine	Thiodiglycol and conjugates (acid hydrolysis)	14·4
pH 11-eluate	Thiodiglycol	1·4
Whole urine	Bis-β-chloroethyl sulfone and conjugates	7·4
Whole urine	Bis-β-chloroethyl sulfone	1·0
Whole urine	Sulfone of thiodiglycolic acid	4·8
Whole urine	Thioglycolic acid and conjugates	1·0
pH 1-eluate	Thiodiglycolic acid	0·2
Whole urine	Bis-β-chloroethyl sulfoxide	0·5
pH 0-eluate	Inorganic sulfate	0·5
pH 0-eluate	Isethionic acid	0·2
pH 0-eluate	Sulfoacetic acid	0·2
Whole urine	Cysteine-bis-β-chloroethyl sulfone conjugate	0·2

Identification of the reaction product of bis-β-chloroethyl sulfone with glutathione

Since it appeared from the earlier studies that a portion of bis-β-chloroethyl sulfide could be oxidized to a sulfone, the reaction product of this sulfone with glutathione was also investigated. This product was a white crystalline substance similar to that described by Hartwell[3]. Carrier amounts were mixed with radioactive urine and repeatedly precipitated with ethanol and acetone to constant specific activity. About 7 per cent of the activity was accounted for as this substance.

The values for all the identified substances are summarized in Table 2.

Compounds not detected in rat urine

An attempt to detect unchanged bis-β-chloroethyl sulfide in urine by extraction into petroleum ether and application of the reaction of Harley-Mason[20] met with no success, a finding which indicated that amounts of the drug in excess of 5 μg are not present.

It seemed possible that one end of the thiodiglycol molecule might have been oxidized to the carboxylic acid, whereas the other end was not. Accordingly, the

lactone of this acid, hydroxyethylmercaptoacetic acid was prepared according to Hellström[21]. Attempts to isolate this product from rat urine were unsuccessful. Likewise, an attempt to synthesize hydroxyethylsulfonyl acetic acid[22] was unsuccessful. Since the substances seemed of minor interest, these experiments were abandoned.

DISCUSSION

Fig. 3 presents the structure of the various substances which have been found. The metabolites present in largest amount are thiodiglycol, chiefly as acid-labile

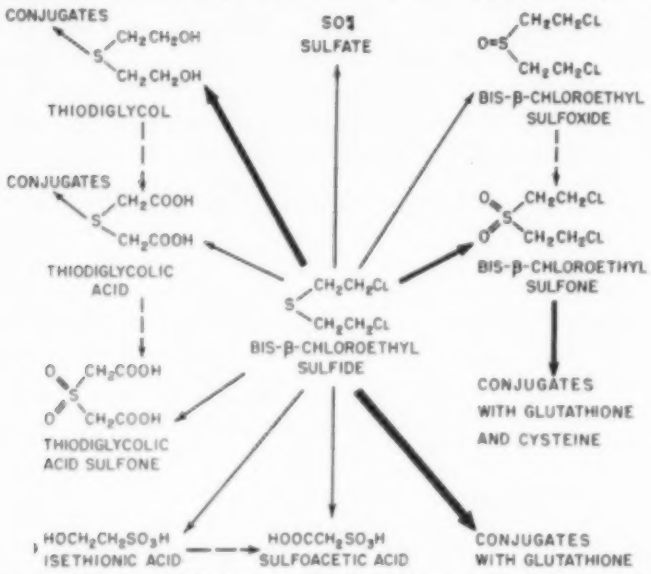


FIG. 3. Structures of the identified urinary metabolites. Heavy arrows indicate major pathways. Dashed arrows indicate possible pathways.

conjugates, and one or more reaction products with glutathione. Since the latter substances might very well be hydrolyzed to thiodiglycol, the two types are not mutually exclusive. In a similar way, conjugates of bis-β-chloroethyl sulfide with proteins, nucleic acids, etc., might also be hydrolyzed to thiodiglycol. The formation of thiodiglycol may very well be non-biochemical in origin, since it is well known that bis-β-chloroethyl sulfide hydrolyzes extensively within minutes in water. As shown in Fig. 2, thiodiglycol itself is metabolized within the mammalian body and yields a peak at pH 11, two peaks emerging at pH 5 and small amounts of highly acidic substances. The finding of trace amounts of sulfate and the less highly oxidized substances, sulfoacetic acid and isethionate, might be merely an indication that some of the thiodiglycol formed is partially or completely oxidized, both at the central sulfur atom and at the terminal carbon atoms. Because of the highly water-soluble nature of thiodiglycol and its conjugates, however, there is apparently little physiological requirement for such oxidation. The failure to detect significant amounts of sulfate rules out any extensive conversion of the drug to hydrogen sulfide, since the mammalian body oxidizes hydrogen sulfide to sulfate.[23]

The evidence suggests that a portion of the drug is oxidized to sulfones, possibly by way of sulfoxides, since two different sulfones and several conjugates have been

detected. These results are in agreement with the finding of the sulfoxide as a metabolite of promazine[24] and of methyl ethyl sulfone as a metabolite of ethanethiol.[25]

Since Kinsey and Grant[26] have shown that up to 50 per cent of bis-β-chloroethyl sulfide reacting with yeast does so with glutathione, the apparently large amount conjugated here is not unexpected. The finding of larger amounts of conjugates of sulfur mustard with glutathione than with cysteine is probably merely indicative of the relatively higher concentration of glutathione in plasma. It is reported that whole rat blood contains 40 mg of reduced glutathione per 100 ml, and red cells even more. This is far in excess of the amount required to react with the dose of drug given. It must be recalled that the glutathione conjugate is not a well-defined substance. In contrast, cysteine is present to the extent of only 0·3 mg per cent in blood. The failure to find appreciable amounts of cysteine conjugates also suggests that if these drugs do react with proteins through the sulfhydryl group, these proteins are not hydrolyzed completely prior to excretion or that they are retained for long periods. The relatively constant level of radioactivity in blood favors the latter viewpoint.

The major metabolites so far identified seem to reveal little with regard to the mechanism of action of the drug since they reflect simply hydrolytic processes, or perhaps an immediate non-specific reaction with glutathione in the circulatory system. There remain large amounts of radioactivity unaccounted for, much of it in highly acidic forms. The nature of conjugates with other amino acids, purines, pyrimidines, etc., is being undertaken by investigations *in vivo*.

REFERENCES

1. F. E. ADAIR and H. J. BAGG, *Ann. Surg.* **93**, 190 (1931).
2. Z. M. BACQ, *Bruxelles Med.* **27**, 2111 (1947).
3. J. L. HARTWELL, *J. Nat. Cancer Inst.* **6**, 319 (1946).
4. J. C. BOURSNELL, G. E. FRANCIS and A. WORMALL, *Biochem. J.* **40**, 737 (1946).
5. T. E. BANKS, J. C. BOURSNELL, G. E. FRANCIS, F. H. HOPWOOD and A. WORMALL, *Biochem. J.* **40**, 745 (1946).
6. G. P. WHEELER, J. S. MORROW and H. E. SKIPPER, *Arch. Biochem. Biophys.* **57**, 133 (1955).
7. C. STORA, J. BRIGANDO, O. SCHARTZKOPF and H. GENIN, *C. R. Acad. Soc., Paris* **224**, 1367 (1947).
8. J. C. BOURSNELL, G. E. FRANCIS and A. WORMALL, *Biochem. J.* **40**, 743 (1946).
9. M. FRANCHIMONT, *C. R. Acad. Sci., Paris* **92**, 1053 (1881).
10. W. M. LAUER and A. HILL, *J. Amer. Chem. Soc.* **58**, 1873 (1936).
11. O. B. HELFRICH, *J. Amer. Chem. Soc.* **42**, 1208 (1920).
12. R. ANSCHÜTZ and H. IMMENDORFF, *Ber. dtsch. chem. Ges.* **17**, 2818 (1884).
13. N. W. PIRIE, *Biochem. J.* **26**, 2041 (1932).
14. P. K. SMITH, M. V. NADKARNI, E. G. TRAMS and C. DAVISON, *Ann. N. Y. Acad. Sci.* **68**, 834 (1958).
15. J. C. BOURSNELL, J. A. COHEN, M. DIXON, G. E. FRANCIS, G. D. GREVILLE, D. M. NEEDHAM and A. WORMALL, *Biochem. J.* **40**, 756 (1946).
16. S. M. MCELVAIN, *The Characterization of Organic Compounds*, p. 199. Macmillan, New York (1947).
17. A. E. CASHMORE, *J. Chem. Soc.* **123**, 1738 (1923).
18. P. B. HAWK, B. L. OSER and W. H. SUMMERSON, *Practical Physiological Chemistry*, 13th ed., p. 949. Blakiston, New York (1954).
19. S. M. MCELVAIN, *The Characterization of Organic Compounds*, p. 189. Macmillan, New York (1947).
20. J. HARLEY-MASON, *J. Chem. Soc.* 146 (1952).
21. H. HELLSTRÖM, *Svenck. Kem. Tid.* **49**, 201 (1937).
22. J. -A. REUTERSKIÖLD, *Studien uber 1-2-di-sulfid-di-karbonsäuren, Die Entsprechenden di-sulfone und deren spaltungsprodukte*, p. 10. Appelbergs Boktryckeriaktiebolag, Uppsala (1939).
23. A. I. GUNINA, *Bull. Exp. Biol. Med.* **43**, 176 (1957).
24. S. WALKENSTEIN and J. SEIFTER, *J. Pharmacol.* **125**, 283 (1959).
25. G. A. SNOW, *Biochem. J.* **65**, 77 (1957).
26. E. KINSEY and W. M. GRANT, *J. Cell. Comp. Physiol.* **29**, 289 (1947).

Biochemical Pharmacology, 1961, Vol. 7, pp. 75–80. Pergamon Press Ltd., Printed in Great Britain.

SHORT COMMUNICATIONS

Increased levels of folic acid reductase as a mechanism of resistance to amethopterin in leukemic cells

(*Received* 15 *February* 1961)

STUDIES concerning amethopterin-resistance in murine leukemic cells (L5178Y) have been described.[1] Using a medium developed to permit the clonal reproduction of single isolated leukemic cells,[2] it has been established that independently derived "first-step" mutants (presumed to be the result of single independent mutational events), had separate "low" levels of resistance. The concentration of amethopterin required for 50% inhibition of growth of 20 first-step mutant clones ranged from 1·4 to 4·3 $\times$ 10^{-8} M, or 1·5 to 4·8 times that concentration (0·9 $\times$ 10^{-8} M) required for comparable inhibition of the parent strain. This finding suggests that, with respect to this enzymic activity, a multiplicity of mutable sites or alternate stable forms of a single mutable site may be present in these cells. With one such first-step mutant clone, selected in an amethopterin-containing medium which completely prevented the reproduction of sensitive cells, approximately twice the concentration of amethopterin ("2-fold mutant") was needed for 50% inhibition of growth, as was the case with the parent strain. From this first-step clone, a "second-step" clone ("16-fold mutant") was isolated. These first and second-step clones had a 2- and 17-fold increase, respectively, in their levels of folic acid reductase activity (Table 1).[3] Increased folic acid reductase activity has been reported in amethopterin-resistant cells from (a) sublines of mouse leukemia L5178 selected in culture[1, 3] (b) various leukemic lines selected in mice,[7] (c) cultured sarcoma-180 cells,[8] and (d) human leukemic individuals treated with the drug.[9]

TABLE 1. FOLIC ACID REDUCTASE LEVELS OF AMETHOPTERIN-SENSITIVE AND AMETHOPTERIN-RESISTANT CELLS

Line tested	Relative level of resistance*	Relative activity of folic acid reductase†
Sensitive	1·0	1·0
"First-step" clone	2·3	2·0
"Second-step" clone	16·0	17·0

A mixture containing 0·02 M phosphate buffer (pH 6·0), 1·8 $\times$ 10^{-2} M isocitric acid, 1·8 $\times$ 10^{-2} M magnesium chloride, 2 $\times$ 10^{-4} M triphosphopyridine nucleotide and a preparation of isocitric dehydrogenase[3] was incubated for 5 min at 37 °C. Of this mixture, 0·15 ml was added to 0·30 ml of a preparation of folic acid reductase (this enzyme was prepared by the addition of 4 volumes of water to packed cells from culture, dialysis for 18 hours against 1,000 volumes of 0·002 M phosphate buffer (pH 6·0), centrifugation at 100,000 g for 30 min, recovery of the supernatant fraction, and adjustment of the phosphate concentration to 0·02 M (pH 6·0)); the, 0·05 ml of 0·0012 M folic acid was added. After 20 min the reaction was terminated by the addition of 0·2 ml of 4 N hydrochloric acid, 0·8 ml of acetone and 0·5 ml of water. The diazotizable product was determined by the method of Bratton and Marshall.[4]

* Concentration required for 50% inhibition of sensitive line = 0·9 $\times$ 10^{-8} M.

† Activity of sensitive line = 2·6 units of folic acid reductase activity per mg of protein.[5] One unit of folic acid reductase activity = 1 $\times$ 10^{-3} μmoles of product (measured as diazotizable amine) per 30 min.

"Pseudo-irreversible"[10] complete inhibition of one unit of folic reductase activity (see Table 1) required the same amount of amethopterin ($2{\cdot}9 \times 10^{-13}$ moles) whether the enzyme was prepared from drug-sensitive or drug-resistant cells. Since amethopterin is bound to the catalytic site of folic acid reductase,[11, 12, 13] and apparently to no other cell component, it is suggested that an increased number of such catalytic sites, with identical turnover number and "affinity" for amethopterin, are present in the resistant cells.

Resistant cells, grown in the presence of tritium-labeled amethopterin (103 μc/mole) bound the antagonist in a manner which suggested that the entry of amethopterin into the cell was less rapid than that of the synthesis of folic acid reductase (Table 2). From such "bound" enzyme, a minimum of 80% of the ^{3}H-amethopterin was liberated by dialysis against 100 volumes of 0·2 M phosphate buffer (pH 6·0) and enzyme activity was regenerated. Although the amount of bound radioactivity (sufficient for the binding of 8 units of enzyme activity per 10^7 cells) which was release by dialysis was not in close agreement with the units of enzyme recovered after dialysis (15 units per 10^7 cells), it is conceivable that, under the conditions of the experiment, some tritium may have been lost from the amethopterin. In any case, the increased levels of enzyme activity found in the resistant lines correlated directly with the degree of resistance, a circumstance which appears to reflect an increased amount of enzyme synthesis in the resistant cells. It is suggested that such synthesis provides excess enzyme which "binds" nearly all of the amethopterin which is capable of entering the leukemic cells under the conditions described. The enzyme which remains "unbound" under these circumstances is sufficient to reduce folic acid to coenzyme forms essential for cell reproduction.

TABLE 2. BINDING OF FOLIC ACID REDUCTASE DURING THE REPRODUCTION IN CULTURE OF A "16-FOLD" AMETHOPTERIN-RESISTANT MUTANT

Molar concentration ($\times 10^{-8}$) of ^{3}H-amethopterin in growth medium	cpm/10^7 cells		Units of enzyme/10^7 cells	
	cpm	Indicated units bound	Before dialysis	After dialysis
14	290	11		
7·0	190	8	1·6	17
3·5	160	6		
1·8	80	3		
0·9	20	0·8		
0·0	0	0	21	20

For each level of ^{3}H-amethopterin (103 μc/μmole), $2{\cdot}5 \times 10^7$ cells were collected after seven generations in culture. The enzyme was prepared from washed cells and the radioactivity was measured in a liquid scintillation counter. The enzyme activity, expressed in units (see Table 1), was measured by determination of the product formed after incubation for 10 min at varying concentrations of the enzyme. The inhibited enzyme was regenerated by equilibrium-dialysis against 100 volumes of 0·2 M phosphate buffer (pH 6·0) for 18 hr.

In the absence of amethopterin the resistant lines have retained their levels of resistance quantitatively for a period of 6 months. Furthermore, no gross chromosomal abnormalities have been detected in the resistant lines, when these were compared with the near-diploid clone of origin. Comparative nutritional studies of various aspects of the metabolism of folic acid have disclosed no other differences between amethopterin-sensitive and -resistant cells. Thus, the requirement for very high levels of folic acid, which characterizes the drug-sensitive cells,[1, 14] remains elevated in the amethopterin-resistant cells. Also, in the presence of very high levels of amethopterin (10^{-6} M), the same levels of thymidine, hypoxanthine and serine are required to support the growth of either the sensitive or the resistant cells. These findings suggest that the increase in folic acid reductase activity may reflect a single mutational event, associated with a single biochemical alteration in the resistant cell (possibly affecting the extent to which a single enzyme is formed).

Department of Pharmacology
Yale University School of Medicine,
New Haven 11, *Connecticut*

GLENN A. FISCHER

Vol. 7
1961

REFERENCES

1. G. A. Fischer, *Cancer Res.* **19**, 372 (1959).
2. E. E. Haley, G. A. Fischer and A. D. Welch, *Cancer Res.* In press.
3. A. L. Grafflin and S. Ochoa, *Biochim. Biophys. Acta* **4**, 205 (1950).
4. A. C. Bratton and E. K. Marshall, *J. Biol. Chem.* **128**, 537 (1939).
5. L. C. Mokrasch and R. W. McGilvery, *J. Biol. Chem.* **221**, 909 (1956).
6. G. A. Fischer, *Proc. Am. Assoc. Cancer Res.* **3**, 111 (1960).
7. D. K. Misra, S. R. Humphreys, M. Freidkin, A. Goldin and E. J. Crawford, *Nature* **189**, 39 (1961).
8. M. T. Hakala, S. F. Zakrzewski and C. A. Nichol, *Proc. Am. Assoc. Cancer Res.* **3**, 115 (1960).
9. J. R. Bertino, F. M. Huennekens and B. W. Gabrio, *Clin. Res.* **9**, 103 (1961).
10. W. W. Ackerman and V. R. Potter, *Proc. Soc. Exp. Biol. Med.* **72**, 1 (1949).
11. S. F. Zakrzewski and C. A. Nichol, *J. Biol. Chem.* **235**, 2984 (1960).
12. J. Peters and D. M. Greenberg, *Biochim. Biophys. Acta* **32**, 273 (1959).
13. W. C. Werkheiser, *Proc. Am. Assoc. Cancer Res.* **3**, 72 (1959).
14. G. A. Fischer and A. D. Welch, *Science* **126**, 1018 (1957).

The inhibition of norepinephrine and epinephrine synthesis *in vitro*

(*Received* 13 *March* 1961)

The biogenesis of norepinephrine involves the oxidation of phenylalanine to tyrosine, the catalyzed conversion of tyrosine to 3,4-dihydroxyphenylalanine by tyrosinase, the decarboxylation of 3,4-dihydroxyphenylalanine to dopamine (3,4-dihydroxyphenylethylamine) by DOPA decarboxylase, and the β-hydroxylation of dopamine to norepinephrine by dopamine β-oxidase. Epinephrine is formed from norepinephrine by N-methylation of the latter. However, the β-hydroxylation of epinine (3,4-dihydroxyphenylethylmethylamine) may be considered as another possible route for the formation of epinephrine.[1]

The enzyme dopamine β-oxidase, which converts dopamine to norepinephrine, has been shown to be non-specific for dopamine.[5] Among other structural analogs of dopamine, epinine in high concentrations inhibits the conversion of dopamine to norepinephrine. The present communication will show that dopamine β-oxidase catalyzes the conversion of epinine to epinephrine, but at a much lower efficiency than at which dopamine is converted to norepinephrine. Under the same conditions in which dopamine is converted to norepinephrine to the extent of 40–50 per cent, only 5–10 per cent of epinephrine is formed from epinine. It will also be shown that amphetamine and *p*-hydroxyamphetamine inhibit both the conversion of dopamine to norepinephrine and the conversion of epinine to epinephrine.

The enzyme dopamine β-oxidase was prepared by the method of E. Y. Levin *et al.*[2], but the purification on calcium phosphate gel was omitted. Either dopamine or epinine were added in the same concentrations to a mixture which contained the following components (in μmoles): potassium phosphate buffer, pH 6·4, 100; (1-methyl-2-phenyl)-ethyl hydrazine hydrochloride, 1·3; ascorbic acid, 6; fumaric acid, 10; ATP, 12·5. To this mixture, 0·2 ml of the enzyme was added and the final volume was adjusted to 1 ml with phosphate buffer, pH 6·4. The reaction mixture was incubated for 20 min at 37 °C, using air as a gas phase. At the end of the period of incubation the reaction was stopped by the addition of 0·5 ml of 3% trichloroacetic acid, the precipitated proteins were removed by centrifugation, and the supernatant fluid was adjusted to pH 6 and diluted to 10 ml with water. Depending upon the precursor used, an aliquot was then analyzed either for epinephrine or norepinephrine, by a modification of the fluorometric method of von Euler.[3] The compounds used in the present experiments have not been found to interfere with the specificity of the trihydroxyindole fluorometric method. However, an excess of potassium ferricyanide was ued in order to oxidize the ascorbic acid which was present in the incubation mixture.[4] The relative inhibition rate of dopamine β-oxidase by amphetamine and *p*-hydroxyamphetamine was determined by a comparison of the

amount of norepinephrine or epinephrine formed in an incubation mixture which contained only the substrate (dopamine or epinine) and an incubation mixture which contained the compound to be tested and the substrate.

The accompanying table shows the effects of test compounds on the conversion of dopamine to norepinephrine and epinine to epinephrine. *p*-Hydroxyamphetamine is a more potent inhibitor than amphetamine. This is in agreement with the previous findings that tyramine is a more potent inhibitor

TABLE 1. INHIBITION OF NOREPINEPHRINE AND EPINEPHRINE SYNTHESIS *in vitro**

Inhibitor	Amount added in μmoles		Amount formed in μmoles		Percent inhibition	
	Substrate†	Inhibitor	Epinephrine from epinine	Norepinephrine from dopamine	Epinephrine from epinine	Norepinephrine from dopamine
None	1·05	none	0·075 ± 0·01	0·45 ± 0·05		
DL-Amphetamine	1·05	2·75	0·037 ± 0·004	0·315 ± 0·03	50	30
DL-Amphetamine	1·05	5·50	0·018 ± 0·002	0·180 ± 0·02	75	60
DL-*p*-Hydroxyamphetamine	1·05	2·37	0·022 ± 0·002	0·225 ± 0·02	70	50
DL-*p*-Hydroxyamphetamine	1·05	4·75	0·015 ± 0·002	0·135 ± 0·01	80	70

* Figures represent averages of 3 experiments in each series.
† Dopamine or epinine.

for dopamine to norepinephrine conversion than is phenylethylamine.[5]

Studies are now under way on the nature of the inhibition of epinephrine and norepinephrine synthesis by amphetamine and *p*-hydroxyamphetamine, as well as on the extent to which epinine is converted to epinephrine *in vivo*.

Acknowledgements—This work was supported by grants from the National Institutes of Health.

Neurochemistry Laboratory,
Department of Psychiatry and Neurology,
New York University College of Medicine

M. GOLDSTEIN
J. F. CONTRERA

REFERENCES

1. W. BRIDGERS. To be published,
2. E. Y. LEVIN, B. LEVENBERG and S. KAUFMAN, *J. Biol. Chem.* **235**, 2080 (1960).
3. U. S. VON EULER and I. FLODING, *Acta Physiol. Scand.* **33**, 45 (1955).
4. M. GOLDSTEIN *et al.* To be published.
5. M. GOLDSTEIN and J. CONTRERA, *Experientia.* To be published.

Effect of colchicine on the intestinal xanthine oxidase

(*Received* 15 *March* 1961)

COLCHICINE was shown to inhibit the enzyme xanthine oxidase (XO) of rat liver and to enhance the activity of this enzyme in blood serum.[1]

Since the small intestine of the rat is relatively rich in XO,[2] and since colchicine is eliminated through the bile and the bowel, it seemed worthwhile to investigate the effect of this drug on the intestinal XO.

Male Wistar white rats were used in this study. For intestinal analysis, a portion corresponding roughly to the jejunum of the rat was slit, washed thoroughly in running water and blotted dry. It was then analyzed for XO activity by the same general procedure used for liver.[3] The results are expressed in μl O_2/g dry weight/60 min.

Colchicine was administered by injection (0·1 mg/100 g of body weight) as an alcoholic solution (6·8 per cent) or given *ad libitum* orally by adding colchicine Houdé to the diet (5 mg/100 g of diet). Control animals injected with the alcoholic solution (6·8 per cent) without colchicine were simultaneously examined.

Three animals injected with a single dose of colchicine presented values of 1794 $\pm$ 162 μl O_2/g dry weight 22 hr after the injection. Control rats (5 animals) showed almost the same values as normal non-injected rats (1487 $\pm$ 249 μl O_2/g dry weight/60 min). When three doses (0·1 mg/100 g of body weight) were injected (6 rats) during subsequent days, values of 970 $\pm$ 191 were obtained against 1642 $\pm$ 381 for the controls (7 rats).

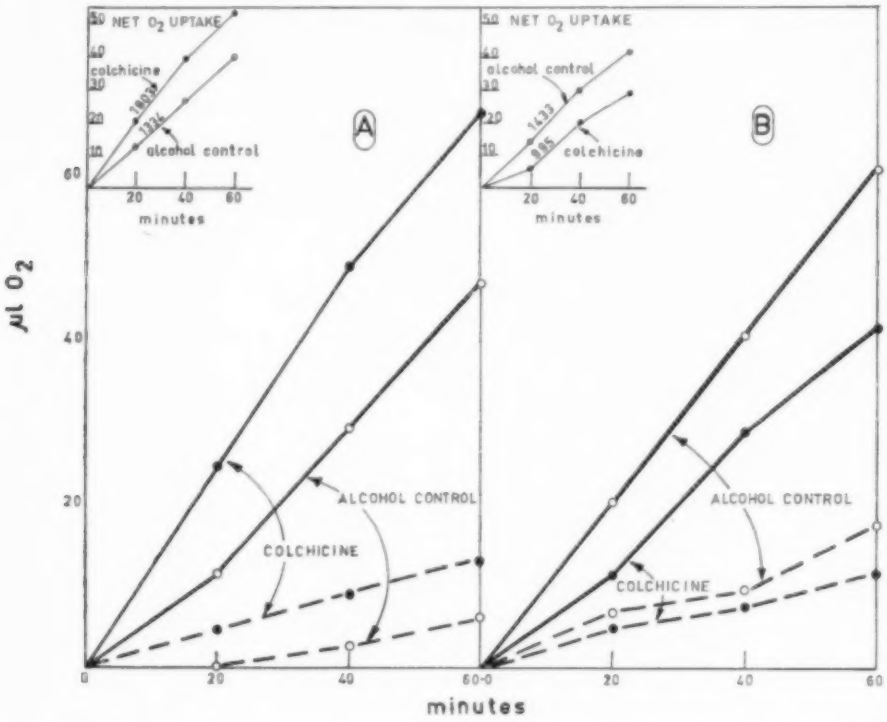


FIG. 1. Effect of colchicine on intestinal xanthine oxidase activity. (A) with a single dose; (B) with three doses. The numbers in graph in the upper part represent values of net O_2 uptake in μl O_2/g dry weight/60 min.

These results show that colchicine, injected as a single dose, slightly increases the activity of the intestinal XO ($t = 2{\cdot}41$, $0{\cdot}02 < P < 0{\cdot}05$). However, the enzyme is inhibited when the drug is administered orally (5 mg/100 g of diet) or when three consecutive doses are injected (0·1 mg/100 g of body weight), $t = 2{\cdot}87$, $0{\cdot}02 < P < 0{\cdot}05$, as shown in Fig. 1.

Acknowledgements—This investigation was supported in part by grants from the Conselho Nacional de Pesquisas (O. R. Affonso and E. Mitidieri).

Biochemical Laboratory,
Instituto Oswaldo Cruz,
Rio de Janeiro, Brazil

G. G. Villela
O. R. Affonso
E. Mitidieri

REFERENCES

1. O. R. Affonso, E. Mitidieri and G. G. Villela. In press.
2. W. W. Westerfeld, D. A. Richert and M. F. Hilfinger, *Cancer Res.* **10**, 486 (1950).
3. G. G. Villela and E. Mitidieri, *Nature* **175**, 208 (1955).

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 81–87. Pergamon Press Ltd., Printed in Great Britain.

THE INFLUENCE OF HALOPERIDOL AND CHEMICALLY RELATED NEUROLEPTICS ON FREE AMINO ACID CONTENT OF RAT BRAIN

HANS SOEP and PAUL A. J. JANSSEN

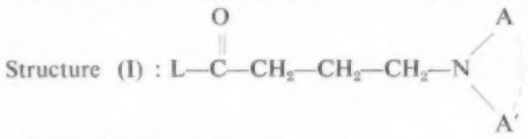
Research Laboratory Dr. C. Janssen, Beerse, Belgium

(*Received* 23 *November* 1960)

Abstract—The influence of a single subtoxic subcutaneous dose of Haloperidol and of three other neuroleptic drugs of the butyrophenone series on the concentrations of a series of free amino acids occurring in rat brain has been studied. Various significant effects were found. It is doubtful however whether these effects are related to the biochemical mechanism of action of these compounds. A peptide occurring in normal rat brain was found to contain seven different amino acids.

INTRODUCTION

IN RECENT years a large number of basic butyrophenones of general structure (I) were synthesized and investigated pharmacologically in this laboratory.



Structure (I) : L—C(=O)—CH_2—CH_2—CH_2—NAA′

L = phenyl, substituted phenyl or 2-thienyl.

NAA′ = heterocyclic tertiary amine, such as 4-substituted piperidine or 4-substituted piperazine.

It was found that many of these compounds display strong and typical neuroleptic activity. Some of them are powerful blockers of epinephrine. Their properties are, generally speaking, rather similar to those of chlorpromazine and related phenothiazines.[1–7]

For a number of reasons we became interest in exploring the possible interactions between these butyrophenones and gamma amino butyric acid (GABA):

(1) As shown in Table 1, the structural requirements for GABA-like activity on the one hand and neuroleptic activity on the other hand are strikingly similar.

(2) GABA is known to play an important role as an inhibitor of the central nervous system, particularly of the mid-brain, while neuroleptics must also be regarded as inhibitors of mid-brain function.[18, 19]

The purpose of this paper is to present preliminary results obtained from experiments designed to analyse the influence of various butyrophenones of structure (I) on the concentrations of free amino acids in rat brain.

METHODS

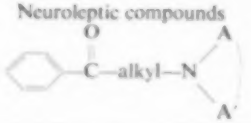
1. *Preparation of the brain extracts*

Untreated male rats of an inbred Wistar strain were used in all the experiments. The pharmacological compounds under investigation were injected subcutaneously

and 4 hr later the rats were killed by decapitation. Control rats, injected with the same amount of solvent were killed at the same time.

The animals weighed around 300 g and were approximately 2½ months of age. The average weight of the brain as determined immediately after removal was 1·6 g. The brains of two rats, injected with the same compound, were pooled and immediately put in 12·8 ml of 75% ethanol per g fresh tissue. Tissue extracts were prepared free of proteins and lipids as described by Roberts[8].

TABLE 1. STRUCTURAL REQUIREMENTS OF GABA-LIKE ACTIVITY AND OF NEUROLEPTIC ACTIVITY

Alkyl	GABA-like compounds HO—C(=O)—alkyl—NH_2		Neuroleptic compounds 	
	Name	Activity*	Name	Activity†
$—CH_2—$	glycine	+ (1/1300)	ω-aminoacetophenones	+ (<1/100)
$—(CH_2)_2—$	β-alanine	+ + + (1/20)	β-aminopropiophenones	+ + + (1/100)
$—(CH_2)_3—$	GABA	+ + + + + + (1)	γ-aminobutyrophenones	+ + + + + + (1)
$—(CH_2)_4—$	δ-aminovaleric acid	+ + + + + (1/15)	δ-aminovalerophenones	+ + + + + (1/10)
$—(CH_2)_5—$	ε-aminocaproic acid	+ + + + (1/100)	ε-aminocaprophenones	+ + + + (1/50)
$—CH_2—CH(CH_3)—$	β-aminobutyric acid	+ + (1/500)	β-aminobutyrophenones	+ + (<1/100)

* Edwards.[17]
† Janssen *et al.*[3–5]

The method of Roberts was slightly modified. The aqueous ethanol extract was evaporated almost to dryness, taken up in 5–10 ml water and centrifuged for 60 min at 6000 rev/min (swing-out heads). The supernatant was again evaporated to ±0·5 ml. By adding water a final volume of 1 ml was obtained.

2. *Chromatography*

Two-dimensional descending chromatography was used. Twenty microlitres of the extract (an aliquot corresponding to 65 mg fresh brain tissue) are placed on Whatman no. 1 paper (35 cm × 35 cm). The first solvent was a mixture of 80 g melted phenol, 20 g water and 10 mg EDTA. After the phenol run, the papers were dried for 1 hr at 65 °C in a "chromatography drying oven" with forced air circulation.

The second solvent was the supernatant of 1 vol. of collidine, 1 vol. of lutidine and 2 vols. of water. The sheets of paper were dried for 30 min at 65 °C. Even better

separation of amino acids was obtained when the papers were first run with phenol and dried at 65 °C before the amino acid mixture was applied.*

3. *Colour development*

The spots were visualized by dipping each sheet of paper in 50 ml of 1% ninhydrin solution in acetone for 10 sec, followed by drying for 30 min at 65 °C.

4. *Elution of the spots*

Quantitative determinations of the coloured spots were performed by cutting out equal strips from the chromatograms and transferring them into test tubes with 1 ml, 2 ml, 5 ml or 10 ml of a 71% methanol solution in water according to the intensity of the spot.[9] Half an hour later the tubes were shaken for 10 sec and the intensity of the colour was measured by differential colorimetry.

5. *Identification of a peptide*

Several spots of a peptide found on different chromatogram were cut out under a u.v. lamp after the papers were heated for 15 min at 90 °C. After eluting the peptides from the pieces of paper by dipping in warm water, the volume of the aqueous extract was reduced to ±0·5 ml by evaporation on a warm water bath. The peptide solution was transferred in a small pressure tube and hydrolysed with the same volume of hydrochloric acid for 24 hr at 110 °C. The tube was opened, HCl evaporated and a two-dimensional chromatogram run with and without addition of known amino acids.

RESULTS

A solution was prepared which contained 10 μmole/ml of aspartic acid, glutamic acid, α-alanine and γ-aminobutyric acid. The amino acids of this mixture were separated by the method as previously outlined.

The absorbances of the eluates from the spots were plotted against the micromoles of amino acid applied to the chromatogram. Fig. 1 shows that Beer's law is followed in the range studied.

Determinations of the concentrations of aspartic acid, glutamic acid, α-alanine and γ-aminobutyric acid per 100 mg fresh brain tissue gave the following results:

Compound	Pairs of rats	Concentration m-mole/100 g fresh brain Average	Range
Aspartic acid	10	0·34	0·30 – 0·38
Glutamic acid	10	1·06	0·92 – 1·12
α-Alanine	10	0·084	0·067– 0·11
GABA	10	0·38	0·36 – 0·44

The following free amino acids in brain tissue could be detected: Intense spots were given by glutamic acid, GABA, aspartic acid, taurine and glutamine; Another intense spot was given by the peptide; Glutathion, glycine, α-alanine, serine, threonine, cystine gave spots of weak colour intensity; Only traces of tyrosine, valine, leucine and β-alanine were found. The peptide disappeared after hydrolysis and was found to contain leucine, valine, tyrosine, α-alanine, glutamic acid, glycine and aspartic acid.

* We wish to thank Dr. Wayne Albers of "The National Institute of Health" (Bethesda) for his advice.

The average R_f-values of the peptide in the two solvents used were 0·24 in phenol and 0·07 in collidine–lutidine.

The influence of relatively large doses of R 1625 (20 mpk), R 2498 (10 mpk), R 2028 (80 mpk) and R 1647 (160 mpk) on the concentration of a series of free amino acids in rat brain was investigated as described in the experimental part. The chemical structures and pharmacological properties of the investigated compounds are given in Table 2.

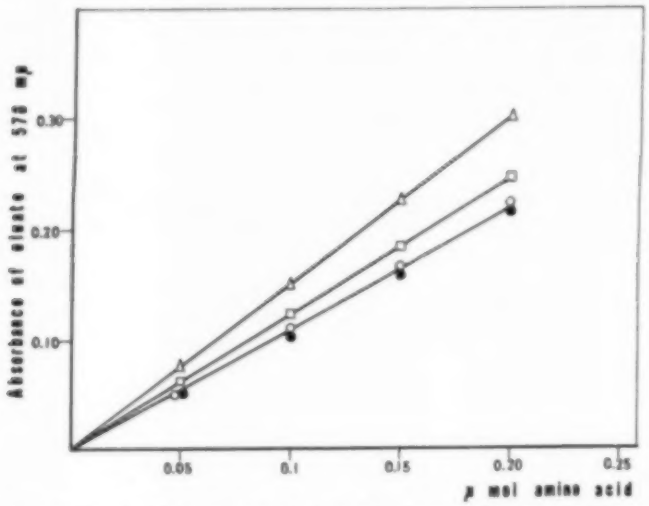
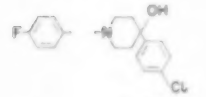
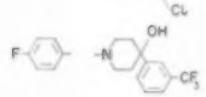
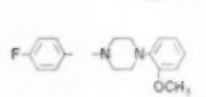
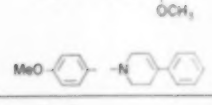


FIG. 1. Absorbance of ninhydrin reaction products as a function of the amount of compound applied to the chromatogram: △ glutamic acid, □ α-alanine, ○ aspartic acid, ● γ-aminobutyric acid. Each mark is the average of five determinations which did not differ more than 10 per cent, respectively.

TABLE 2. CHEMICAL STRUCTURES AND PHARMACOLOGICAL PROPERTIES OF THE INVESTIGATED BASIC BUTYROPHENONES OF STRUCTURE (I)

L– –NAA′	Serial number generic name and references	Neuroleptic activity*	Adrenolytic activity*
	R 1625 Haloperidol[3, 4]	+++	(+)
	R 2498 Triperidol[10]	+++(+)	(+)
	R 2028 Haloanisone[11]	++	+++
	R 1647 Anisoperidone[12]	+	++(+)

* Janssen *et al.*[3–6]

Vol. 7
1961

TABLE 3. INFLUENCE OF SUBSTANCES ON CONCENTRATION OF FREE AMINO ACIDS IN THE BRAIN OF RATS

Amino acid	R 1647		R 2028		R 1625		R 2498	
	$f \pm \epsilon$	$P\%$	$f \pm \epsilon$	$P\%$	$f \pm \epsilon$	$P\%$	$f \pm \epsilon$	$P\%$
Glutamic acid	1·03 ± 0·03	50	1·02 ± 0·05	50	1·00 ± 0·03	50	1·03 ± 0·03	50
GABA	1·10 ± 0·03	6·2	1·01 ± 0·02	50	1·00 ± 0·02	50	1·05 ± 0·04	50
Taurine	1·16 ± 0·07	0·8	1·15 ± 0·05	6·2	1·19 ± 0·06	6·2	1·03 ± 0·05	50
Aspartic acid	1·00 ± 0·04	22·7	1·11 ± 0·07	34·4	1·06 ± 0·06	18·8	1·09 ± 0·07	18·8
Glutamine	1·08 ± 0·08	50	1·23 ± 0·04	0·8	1·14 ± 0·03	0·8	1·11 ± 0·08	50
Glycine	1·20 ± 0·05	0·8	1·11 ± 0·08	22·7	1·18 ± 0·06	6·2	0·94 ± 0·04	18·8
Alanine	1·04 ± 0·05	50	1·05 ± 0·03	18·8	1·00 ± 0·06	50	1·09 ± 0·04	18·8
Threonine	1·04 ± 0·06	50	1·21 ± 0·13	22·7	1·00 ± 0·12	50	1·11 ± 0·10	18·8
Serine	1·22 ± 0·09	10·9	1·06 ± 0·05	50	1·15 ± 0·08	22·7	1·55 ± 0·12	3·1
Valine	1·21 ± 0·06	6·2	1·27 ± 0·06	6·2	1·01 ± 0·08	22·7	1·31 ± 0·07	3·1
Leucine	1·35 ± 0·23	22·7	1·11 ± 0·03	1·6	0·82 ± 0·06	1·6	1·13 ± 0·05	3·1
β-Alanine	0·98 ± 0·10	50	0·85 ± 0·04	6·2	0·93 ± 0·08	10·9	0·91 ± 0·07	50
Glutathione	1·08 ± 0·15	50	1·39 ± 0·08	0·31	1·07 ± 0·18	50	0·94 ± 0·06	50
Peptide	0·94 ± 0·06	50	1·14 ± 0·05	0·31	1·06 ± 0·04	50	1·26 ± 0·08	3·1

The brain extract of both the drug-injected and of the control groups were treated in an identical fashion, 20 μl of each being put on the chromatogram after reduction of the total volume to 1 ml. A total of seven experiments were conducted for each compound. The results are summarized in Table 3, in which the following symbols are used:

(1) $f = \epsilon(x/y)/7$, in which x and y are the extinction values for the treated and the untreated animals, respectively.

(2) ϵ = standard error ($n = 7$) of f.

(3) $P\%$ = the binomial probability of the H_0-hypothesis (one-tailed).

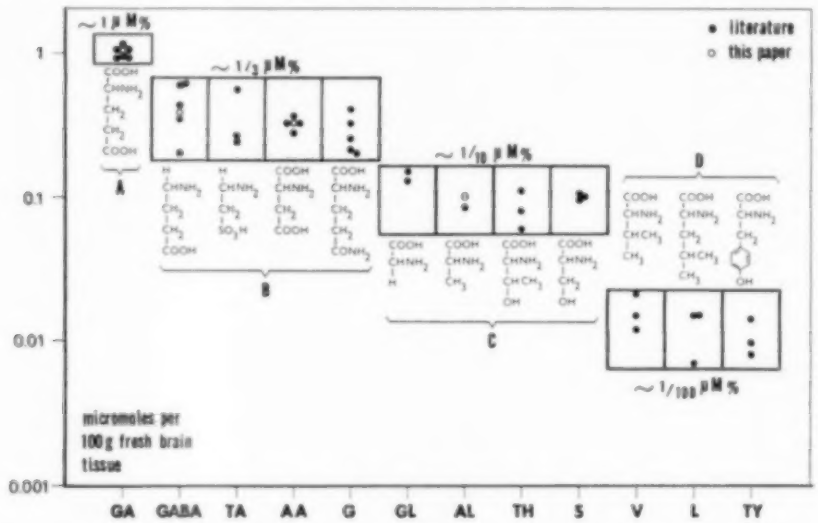


FIG. 2. The table of Porcelatti[13] has been further completed by data from Waelsch[14], Tsukada[15] and Häkkinen[16].

DISCUSSION

As shown in Fig. 2, our findings concerning the free amino acid content (glutamic acid, GABA, aspartic acid and alanine) in the brain of normal laboratory rats, are in excellent agreement with corresponding data from the literature.[13] At least twelve free amino acids occur in rather constant concentration in rat brain. They may be subidivided in four groups, A to D, as shown in Fig. 2. Glutamic acid, the only member of group A, occurs in the amazingly constant concentration of $1{\cdot}0 \pm 0{\cdot}1$ μmole per 100 g fresh tissue. The concentration of the four members of group B are roughly the same and about $0{\cdot}4 \pm 0{\cdot}2$ μmole %. The four amino acids of group C occur at a concentration of about $0{\cdot}10 \pm 0{\cdot}05$ μmole %, and the three acids of group D of about $0{\cdot}013 \pm 0{\cdot}007$ μmole %. It is interesting to speculate on the biochemical mechanisms responsible for this classification. The peptide which is present in normal brain extracts of rats, was found to contain seven amino acids: glutamic acid (group A), aspartic acid (group B), glycine, α-alanine (group C), valine, leucine and tyrosine (group D). The exact structure, the biochemical and neurophysiological significance of this peptide are to be studied in further detail.

Vol. 7
1961

Before trying to interpret the effects of the four neuroleptics studied on the free amino acid concentration of rat brain it should be pointed out that the results of Table 3 were obtained after treatment (4 hr) with a single and very high subtoxic subcutaneous dose of each compound.

In contrast with expectations, GABA was not affected at all after treatment with R 2028, R 1625 or R 2498 and only slightly increased after R 1647.

Glutamic acid, the precursor of GABA, was equally unaffected by all four drugs. Glutamine concentrations, however, were significantly increased after R 2028 and R 1625, but less so after the other two drugs. The other members of group B, i.e. taurine and aspartic acid, also showed a slight tendency to increase, which was significant in some instances. The amino acids of group C were either not influenced or slightly increased in a few cases, i.e. glycine with R 1647 and with R 1625 and serine with R 2498 and possibly with R 1647. A significant increase in valine-concentrations was observed after R 2498, R 1647 and R 2028, whereas leucine was increased after R 2028, R 2498, decreased after R 1625 and not influenced by R 1647. Glutathione, β-alanine and the peptide were also influenced in some instances, particularly by R 2028.

It may be concluded therefore that high doses of all four drugs do have various significant effects on the amino acids in the brain of rats. The whole picture, however, is rather confusing in that the effects vary considerably from compound to compound. This is particularly striking for R 1625 and R 2498. These drugs are very similar pharmacologically and clinically and approximately equi-active. Their effects in the experiments described in this paper, however, are rather different. No useful clue as to a possible relationship between the mechanism of action of these drugs and amino acid metabolism and function can be derived from our experiments. It should be pointed out, however, that our data do not constitute adequate proof of the contrary.

The influence of smaller repeated doses of neuroleptic agents on amino acid metabolism in isolated areas of the brain, e.g. the area postrema, merits further study.

Acknowledgements—We wish to thank Mr. René De Meyer for the excellent technical assistance and Mrs. Annie Claessens for typing the manuscript.

REFERENCES

1. J. R. Boissier, *Acta Neurol. Belg.* **60**, 39 (1960).
2. J. R. Boissier and J. Pagny *et al.*, *Thérapie* **15**, 479 (1960).
3. P. A. J. Janssen *et al.*, *J. Med. Pharm-Chem.* **1**, 281 (1959).
4. P. A. J. Janssen and C. J. E. Niemegeers, *Arzneimittel-Forsch* **9**, 765 (1959).
5. P. A. J. Janssen, A. H. M. Jageneau and K. H. L. Schellekens, *Psychopharmacologia* **1**, 389 (1960).
6. P. A. J. Janssen, A. H. M. Jageneau and C. J. E. Niemegeers, *J. Pharmacol.* **129**, 471 (1960).
7. C. J. E. Niemegeers, Thèse, Sorbonne (1960).
8. E. Roberts and Frankel, *J. Biol. Chem.* **187**, 55 (1950).
9. R. E. Kay *et al.*, *Arch. Biochem. Biophys.* **63**, 14 (1959).
10. P. Divry *et al.*, *Acta Neurol. Belg.* **60**, 463 (1960).
11. P. Paquay *et al.*, *Acta Neurol. Belg.* **60**, 677 (1960).
12. P. Divry *et al.*, *Acta Neurol. Belg.* **59**, 1033 (1959).
13. G. Porcelatti and Thompson, *J. Neurochem.* **1**, 340 (1957).
14. H. Waelsch, *Naturwissenschaften* **15**, 404 (1953).
15. Y. Tsukada *et al.*, *J. Neurochem.* **2**, 295 (1958).
16. H. M. Häkkinen and E. Kulonen, *Nature, Lond.* **184**, 726 (1959).
17. C. Edwards, *Inhibition in the Nervous System and Gamma-amino-butyric Acid*, p. 403. Pergamon Press, Oxford (1960).
18. S. Berl and H. Waelsch, *J. Neurochem.* **3**, 161 (1958).
19. J. Awapara *et al.*, *J. Biol. Chem.* **187**, 35 (1950).

Biochemical Pharmacology, 1961, Vol. 7, pp. 88–95. Pergamon Press Ltd., Printed in Great Britain.

A NEW AND RAPID COLORIMETRIC DETERMINATION OF ACETYLCHOLINESTERASE ACTIVITY

GEORGE L. ELLMAN, K. DIANE COURTNEY, VALENTINO ANDRES, JR. and ROBERT M. FEATHERSTONE

Department of Pharmacology, University of California Medical Center, San Francisco, California

(*Received* 14 *November* 1960)

Abstract—A photometric method for determining acetylcholinesterase activity of tissue extracts, homogenates, cell suspensions, etc., has been described. The enzyme activity is measured by following the increase of yellow color produced from thiocholine when it reacts with dithiobisnitrobenzoate ion. It is based on coupling of these reactions:

$$\text{acetylthiocholine} \xrightarrow{\text{(enzyme)}} \text{thiocholine} + \text{acetate}$$

$$\text{thiocholine} + \text{dithiobisnitrobenzoate} \longrightarrow \text{yellow color}$$

The latter reaction is rapid and the assay is sensitive (i.e. a 10 μl sample of blood is adequate). The use of a recorder has been most helpful, but is not essential. The method has been used to study the enzyme in human erythrocytes and homogenates of rat brain, kidney, lungs, liver and muscle tissue. Kinetic constants determined by this system for erythrocyte cholinesterase are presented. The data obtained with acetylthiocholine as substrate are similar to those with acetylcholine.

INTRODUCTION

A FEW years ago Bonting and Featherstone[1] introduced a modification of the Hestrin hydroxamic acid method[2] suitable for the determination of cholinesterase levels in small quantities of cells cultured *in vitro*. This modification was used successfully in several studies involving the control of enzyme levels in cells by manipulating the levels of substrates or closely related compounds present in the medium.[3] Several interesting areas of research were indicated by these studies. However, this modified method of enzyme assay, although scaled down to the micro-level, had several disadvantages. Among these was the fact that only a terminal figure could be obtained from the material in one tube of cultured cells. Thus, the time course of the reaction could not be followed without resorting to separate experimental tubes for each time interval desired. The method also had the disadvantage that the color measured was developed from the remainder of an added substrate—a procedure in which the possibility of error is relatively great when the level of enzyme activity is small.

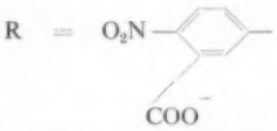
Consideration of the relative merits of various methods which might be useful in studying the time-course of acetylcholinesterase activity in very small tissue samples led us to combine a method reported by Koelle[4] with a sulfhydryl reagent studied by Ellman.[5] This new method, which is presented here, is extremely sensitive and is applicable to either small amounts of tissue or to low concentrations of enzyme. It makes detailed kinetic studies of acetylcholinesterase activity possible. The progress of the hydrolysis is followed by the measurement of a product of the reaction.

Acetylthiocholine is used as the substrate. This analog of the natural substrate has been used most extensively by Koelle[4] for histochemical localization. Other workers[6, 7] have used the sulfur analog in the enzyme assay. Their work, in addition to data we shall present, suggests that this compound is a satisfactory substitute for the natural substrate, and differs much less than some of the synthetic substrates frequently used as in assays of phosphatases, trypsin, chymotrypsin, pepsin, etc.

The principle of the method is the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolyzed. This is accomplished by the continuous reaction of the thiol with 5 : 5-dithiobis-2-nitrobenzoate ion (I)[5]

$$H_2O + (CH_3)_3\overset{+}{N}CH_2CH_2SCOCH_3 \xrightarrow{\text{(enzyme)}} (CH_3)_3\overset{+}{N}CH_2CH_2S^- + CH_3COO^- + 2H^+$$

$$(CH_3)_3\overset{+}{N}CH_2CH_2S^- + \underset{\text{(I)}}{RSSR} \longrightarrow (CH_3)_3\overset{+}{N}CH_2CH_2SSR + \underset{\text{(II)}}{RS^-}$$



to produce the yellow anion of 5-thio-2-nitro-benzoic acid (II). The rate of color production is measured at 412 mμ in a photometer. The reaction with the thiol has been shown to be sufficiently rapid so as not to be rate limiting in the measurement of the enzyme, and in the concentrations used does not inhibit the enzymic hydrolysis. By recording the output of the photometer continuously, records of the complete assay can be obtained (Fig. 1).

We considered it desirable to establish that this method yields results comparable with other procedures. For this reason, the effects of inhibitors were examined; the kinetic constants were calculated and compared with those obtained by other methods. In addition, we were able to compare assays on blood samples by the ferric-hydroxamate method[8] and the present one.

METHODS

The reaction rates were recorded with a Beckman DU spectrophotometer equipped with a Beckman adapter and a Minneapolis–Honeywell recorder.

The general method used was to place buffer in the photocell and add concentrated solutions of reagents by means of micropipettes. The mixture was stirred by continued blowing through the pipettes while moving them around the bottom of the photometer cells. In this way, reagents were added, mixed, and the cover of the cell compartment replaced within 10–15 sec. Our photometer required 30–40 sec to become stabilized to new light conditions. Thus, there was about 1 min when the readings were due to a combination of factors (e.g. bubbles rising through the solutions, sulfhydryl material in the "enzyme", etc.) which were unrelated to the desired measurements. Subsequent readings were strictly dependent on the absorption of the solution under consideration and even rapid changes were followed by the recorder faithfully, as evidenced by the reproducibility of time-transmission curves (Fig. 1).

Solutions

Buffer. Phosphate, 0·1 M, pH 8·0.

Substrate. Acetylthiocholine iodide,* 0·075 M (21·67 mg/ml). This solution was used successfully for 10–15 days if kept refrigerated.

Reagent. Dithiobisnitrobenzoic acid (DTNB) 0·01 M of the 5 : 5-dithiobis-2-nitrobenzoic acid† prepared as described previously,[5] 39·6 mg were dissolved in 10 ml pH 7·0 phosphate buffer (0·1 M) and 15 mg of sodium bicarbonate were added. The reagent was made up in buffer of pH 7 in which it was more stable than in that of pH 8.

Enzyme. Bovine erythrocyte cholinesterase (Nutritional Biochem. Corp., 20,000 units) was dissolved in 20 ml of 1% gelatin. This solution was diluted 1 : 200 with water for use, yielding a solution of 5 units/ml.

General method

A typical run used: 3·0 ml pH 8·0 buffer, 20·0 μl substrate, 100·0 μl DTNB (reagent), 50·0 μl enzyme. The results of several runs are shown in Fig. 1. The blank for such a run consists of buffer, substrate, and DTNB solutions. The absorbances‡ were read from the strip charts and plotted on a rectangular graph paper, the best line drawn through the points and the slope measured. In a run such as that described above, the linear portion of the curve describing the hydrolysis was observed during the first 15–20 min of the reaction; the slope is the rate in absorbance units/min. At this pH level, there is an appreciable non-enzymic hydrolysis of the substrate, and for long runs it was necessary to correct for this. The rate of non-enzymic hydrolysis of acetylthiocholine at 25° was 0·0016 absorbance units per min.

The procedures have been extended to micro-size. A run comparable to those in Fig. 1 was made in a micro-cell (total solution volume was 0·317 ml). The rate was 0·102/min, the same as that determined in the larger cuvettes.

Since the extinction coefficient of the yellow anion (II) is known,[5] the rates can be converted to absolute units, viz.:

$$\text{rate (moles/l. per min)} = \frac{\Delta \text{ absorbance/min}}{1{\cdot}36 \times 10^4}$$

In dealing with cell extracts or suspensions, a blank consisting of extract or suspension, DTNB, and buffer may be required to correct for release of thiol material from the cells and the absorbance of the other materials in the suspension.

Method for blood

A fairly stable suspension was formed from whole blood or washed human erythrocytes. Since the acetylcholinesterase is on the cell membrane, hemolysis was not necessary. The assay of blood was carried out as follows:

(1) A suspension of the blood cells§ in phosphate buffer (pH 8·0, 0·1 M) was prepared. The most practical dilution was 1 : 600 (e.g. 10μl blood into 6 ml buffer).

(2) Exactly 3·0 ml of the suspension were pipetted into a cuvette.

* California Corporation for Biochemical Research, Los Angeles, California.

† This is now available from the Aldrich Chemical Co., 2369 No. 29th, Milwaukee 10, Wisconsin.

‡ Strip charts printed in absorbance units are available from Minneapolis–Honeywell Corporation, Chart 5871.

§ Red cell counts were performed by the clinical laboratory.

Vol. 7
1961

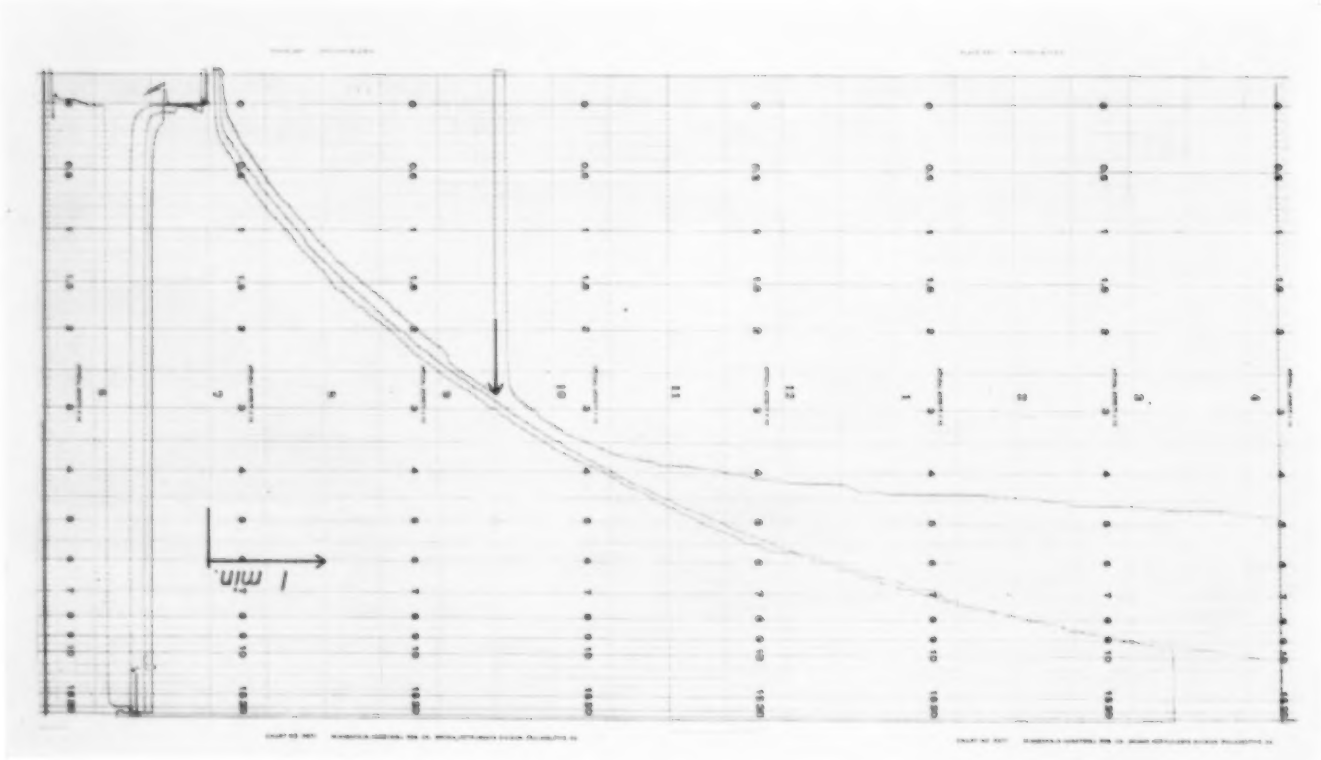


FIG. 1. Photograph of strip chart record of two identical assays. At the arrow, physostigmine salicylate (final concentration, 3×10^{-7} M) was added to a third replicate.

facing page 90

BLANK
PAGE

Vol. 7
1961

(3) If only red cell cholinesterase was desired, 10 μl of 0·1 per cent quinidine sulfate was added to inhibit plasma esterase.[8] If total esterase was desired, this step was omitted. See comment on quinidine in the discussion section.

(4) Of the DTNB reagent, 25 μl were added. The cuvette was placed in the photometer.

(5) The slit of the photometer was adjusted so that the absorbance (at 412 mμ) of the suspension in the cuvette was zero.

(6) Of the substrate, 20 μl were added to this cuvette. Changes in absorbance at 412 mμ were recorded for at least 6 min.

(7) Calculations:

$$\text{moles substrate hydrolyzed/min per R.B.C.} = (4{\cdot}41)\,(10^{-14})\,\frac{\Delta A}{\text{R.B.C.}}$$

where $4{\cdot}41\,(10^{-14})$ = factor for dilution, extinction coefficient, and changes in units;

$$= \frac{600}{13{,}600} \times \frac{1}{10^6\ \text{mm}^3/\text{l.}} \times \frac{1}{10^6\ \text{R.B.C.}};$$

ΔA = change in absorbance/min;
R.B.C. = red cell count (in millions per mm^3).

Other tissues

We have also assayed rat lung, liver, stomach, heart, muscle and brain by this procedure. The steps we used were these:

(1) The tissue was homogenized (approximately 20 mg of tissue per ml of phosphate buffer (pH 8·0, 0·1 M)) in a Potter–Elvehjem homogenizer. For muscular tissue, considerable mincing was necessary before homogenizing.

(2) A 0·4-ml aliquot of this homogenate was added to a cuvette containing 2·6 ml of phosphate buffer (pH 8·0, 0·1 M).

(3) Of the DTNB reagent, 100 μl were added to the photocell. The absorbance was measured at 412 mμ; when this had stopped increasing, the photometer slit was opened so that the absorbance was set to zero.

(4) Of the substrate, 20 μl were added. Changes in absorbance were recorded and the change in absorbance per min. was calculated.

(5) The rates were calculated as follows:

$$R = \frac{\Delta A}{1{\cdot}36\,(10^4)} \times \frac{1}{(400/3120)C_0} = 5{\cdot}74\,(10^{-4})\,\frac{\Delta A}{C_0}$$

where R = rate, in moles substrate hydrolyzed per min per g of tissue;
ΔA = change in absorbance per min;
C_0 = original concentration of tissue (mg/ml).

RESULTS AND DISCUSSION

Reproducibility

The results of repeated assays under various conditions are shown in Table 1. The standard deviations are about 4 per cent of the mean rates, a variability that could be accounted for as pipetting variation.

The rate of hydrolysis of acetylthiocholine by bovine erythrocyte esterase as a function of enzyme concentration is shown in Fig. 2. The unit of enzyme activity is that described by Ammon,[9] and is the amount which hydrolysed 0·01 mg of acetylcholine bromide in 1 min in 3 ml of 0·0092 M acetylcholine bromide. The observed rate was a linear function of enzyme concentration. The data were plotted in terms of the final concentration of enzyme.

TABLE 1. REPRODUCIBILITY OF RATE MEASUREMENTS

Substrate (moles/l.)	Enzyme (units)*	Mean rate†	Standard deviation	Replicates
$5{\cdot}0 \times 10^{-4}$	0·033	0·0402	0·0012	2
$5{\cdot}0 \times 10^{-4}$	0·083	0·1003	0·0038	9
$2{\cdot}5 \times 10^{-4}$	0·083	0·0842	0·0041	4

* Bovine erythrocyte esterase.
† Rate is given in change of absorbance/min. Other details are given under Procedures.

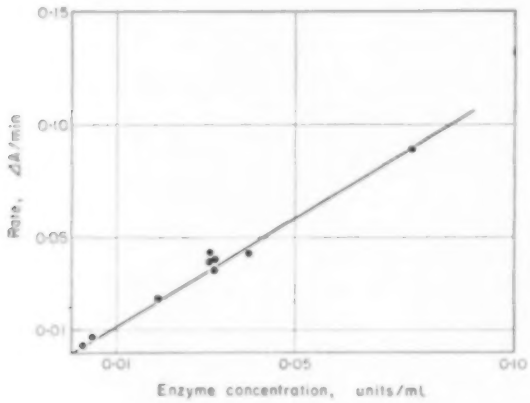


FIG. 2. Rate of thiocholine production as a function of enzyme concentration.

The rate of hydrolysis of acetylthiocholine by bovine erythrocyte cholinesterase as a function of substrate concentration is shown in Fig. 3. The substrate was hydrolysed at an appreciable rate without enzyme above 5×10^{-3} M. The rate plotted for the enzyme is thus the net rate after non-enzymic hydrolysis rate is subtracted from the observed rate. As expected for the "true" enzyme, substrate inhibition was observed, and the data in this experiment were analysed by a reaction sequence that includes this factor. The method of Wright and Sabine[10] was used to analyze these data. The results of the analysis, as well as inhibition constants (also evaluated by the method of Wright and Sabine), are shown in Table 2. The agreement with literature values is close enough that estimates of enzyme rates determined with acetylthiocholine may be considered good estimates of the rate for acetylcholine hydrolysis.

Twelve samples of human blood were assayed by our technique and the ferric hydroxamate method.[8] The comparison is shown in Fig. 4. It is apparent that the observed rates were directly proportional. Thus, the two assays were clearly measuring the same activity. We used quinidine sulfate (2×10^{-5} M, final concentration) to inhibit the plasma esterase.[8] Using the above technique with samples obtained from eighty-seven people, we obtained as the mean and standard deviation ($1{\cdot}08 \pm 0{\cdot}16$) $\times$ 10^{-15} moles substrate hydrolysed per min per red blood cell. Each assay took about 5 min. It is possible to determine at least fifty samples per day.

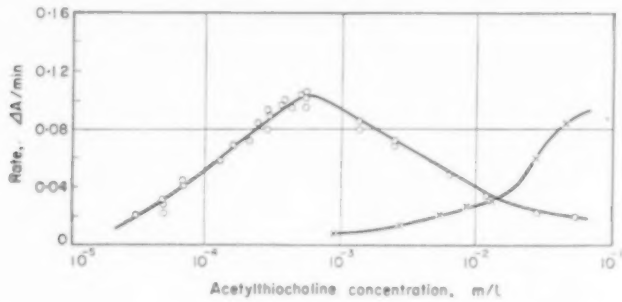


FIG. 3. Rate of thiocholine production as a function of substrate concentration; ○, enzymic ×, non-enzymic.

It should be noted that the concentration of quinidine used will not necessarily inhibit the non-specific esterase of all species or of all tissues (see e.g.[5]). It may, in fact, not be possible to inhibit all the non-specific esterase without inhibiting some of the acetylcholinesterase. It may be necessary, in the study of a given tissue to determine the appropriate concentration of a selective inhibitor (e.g. DFP), in order to use the method for determination of specific acetylcholinesterase.

TABLE 2. KINETIC CONSTANTS OF ERYTHROCYTE CHOLINESTERASE

Constant	Acetylthiocholine	Acetylcholine	Ref.
Michaelis (K_M)	$1{\cdot}4$ (10^{-4})	$2{\cdot}0$ (10^{-4})	10
Inhibition (K_I)			
Substrate	$2{\cdot}9$ (10^{-2})	$1{\cdot}5$ (10^{-2})	10
Decamethonium	$6{\cdot}1$ (10^{-6})	$2{\cdot}5$ (10^{-5})	12
Physostigmine	$2{\cdot}3$ (10^{-8})	$6{\cdot}1$ (10^{-8})	13
Quinidine	$8{\cdot}7$ (10^{-4})	$9{\cdot}6$ (10^{-4})	10

The activity of various rat tissues on acetyl- and butyryl-thiocholine was measured and results are presented in Table 3. These results were obtained in order to establish the applicability of the method, and are not intended to represent a study of these enzymes in these tissues. Since most of these tissues contain non-specific esterases (possibly thiolesterases), the non-cholinesterase hydrolysis might best be determined by incorporating the proper amount of physostigmine in an extra blank to be subtracted.[11]

Vol. 7
1961

The method described here is, in principle, similar to that of Gal and Roth.[7] However, we feel that our method has several advantages: (a) it is dependent on changes in the visible region of the spectrum, so that unusual changes in the absorbance can be checked immediately, e.g. appearance of turbidity, spills on the photocell windows, etc.; (b) since the extinction coefficient of the nitrothiobenzoate ion is 13,600, as against 5140 for acetylthiocholine chloride, there is at least a 2·5-fold potential increase in sensitivity available; (c) measurements of the appearance of products are usually more sensitive than disappearance of substrate; (d) homogenates of tissue do not require any special handling, i.e. precipitation of protein before readings, etc.; (e) the reagents required are commercially available. For these reasons, we believe that our method is more convenient than that of Gal and Roth.

TABLE 3. AVERAGE RATES OF HYDROLYSIS OF ACETYL- AND BUTYRYL-THIOCHOLINE BY RAT TISSUES

Tissue	Rates (moles/l. per min × 10^{-6} per g of tissue)	
	Acetyl	Butyryl
Lung	1·63	0·54
Liver	1·07	0·65
Muscle (thigh)	1·82	0·12
Kidney	0·28	0·21
Brain (whole)	10·31	0·31

The rates given are average values obtained from tissues of four Sprague–Dawley rats.

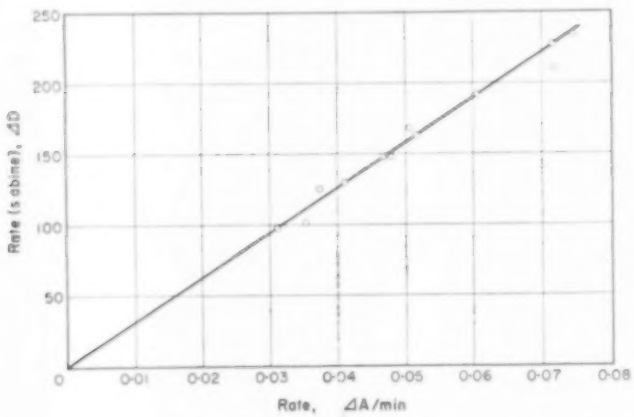


FIG. 4. Comparison of rates of R.B.C. cholinesterase obtained from twelve human R.B.C. samples when the present method and the Sabine[8] method were used.

The hydroxamate method[2] determines the residual substrate after a period of incubation. Hence, only one time period can be measured per analysis, and the execution of kinetic studies with this method becomes difficult. In addition, like the Gal–Roth technique, the disappearance of substrate is being measured. The acidimetric

and gasometric methods, while capable of being used to study kinetics, are inherently less sensitive, often requiring several hundred milligrams of tissue to be useful.

Acknowledgements—We gratefully acknowledge support from the U.S. Public Health Service, National Institutes of Health (Grant CY3861) and State of California Mental Hygiene Commission (Grant 60-1-1302). We wish to thank Dr. J. C. Sabine for her valuable advice and assistance.

REFERENCES

1. S. L. Bonting and R. M. Featherstone, *Arch. Biochem. Biophys.* **61**, 89 (1956).
2. S. Hestrin, *J. Biol. Chem.* **180**, 249 (1949).
3. M. Jones, R. M. Featherstone and S. L. Bonting, *J. Pharmacol.* **116**, 114 (1955).
4. G. B. Koelle, *J. Pharmacol.* **100**, 158 (1951).
5. G. L. Ellman, *Arch. Biochem. Biophys.* **82**, 70 (1959).
6. I. I. A. Tabachnik, *Biochim. Biophys. Acta* **21**, 580 (1956).
7. E. M. Gal and E. Roth, *Clin. Chim. Acta* **2**, 316 (1957).
8. J. C. Sabine, *Blood* **10**, 1132 (1955).
9. R. G. Ammon, *Pflüg. Arch. ges. Physiol.* **233**, 486 (1933).
10. C. I. Wright and J. C. Sabine, *J. Pharmacol.* **93**, 230 (1948).
11. G. B. Koelle, *J. Pharmacol.* **114**, 167 (1955).
12. F. Bergmann, I. B. Wilson and D. Nachmansohn, *Biochim. Biophys. Acta* **6**, 217 (1950).
13. D. Nachmansohn and I. M. Wilson, *Advanc. Enzymol.* **12**, 259 (1951).

ol. 7
961

Biochemical Pharmacology, 1961, Vol. 7, pp. 96–99. Pergamon Press Ltd., Printed in Great Britain.

EFFECTS OF RESERPINE AND COLCHICINE ON ENTEROCHROMAFFIN CELLS*

BERNARD H. MARKS and ROBERT W. SORGEN

Department of Physiological Chemistry and Pharmacology,
The Ohio State University College of Medicine

(*Received* 10 *November* 1960)

Abstract—Enterochromaffin cells can be distinguished in preparations of rabbit duodenal mucosa stained supravitally with neutral red. In reserpinized rabbits, these cells show a loss of granulation in from 4 to 6 hr, corresponding to that previously observed in studies of fixed and stained sections.

Colchicine-treated rabbits also demonstrate a delayed degranulation of enterochromaffin cells, both by the supravital and by the stained section methods. This may have some bearing on the hyperperistalsis known to be produced by colchicine.

Vol. 7
1961

STUDIES of the functional activity and pharmacological responses of the enterochromaffin cell system of the gastroenteric tract are of considerable potential interest. Not only may these cells have a role in regulating peristalsis,[1] but possibly other enteric activities as well, through their synthesis and release of amines, notably 5-hydroxytryptamine (5-HT).[2] Studies of the activity of this cell, occurring singly as they do buried between enteric epithelial cells, have been restricted to methods involving assay of 5-HT activity released from mucosa[3] or counting of enterochromaffin cells in strained sections of gut.[2, 4] These procedures either produce results in terms of statistical averages of cellular activity, or afford indirect estimates of changes in these cells.

In this investigation, the functional response of enterochromaffin cells has been examined directly by observing the disappearance of granules, presumably the repository of 5-HT in these cells. The enterochromaffin cells, which have been located and examined in supravital-stained preparations, have been shown to respond to a 5-HT-releasing drug with the expected loss of staining characteristics.

MATERIAL AND METHODS

All experiments were carried out on young male albino rabbits, weight approximately 3 kg. Animals were injected intravenously with 5 mg of reserpine per kg or with 2 mg of colchicine per kg. Saline-injected controls, sacrificed at the same times as the treated animals, were used.

Segments of duodenum approximately 2 cm long were removed and rinsed gently in saline. The segment was cut lengthwise and spread open upon a glass plate with the mucosa upward. The mucosa was separated from the rest of the strip by scraping

* Supported by Grant RG-6206, National Institutes of Health. Reported in part at the Fall Meeting of the Ohio Valley Section, Society for Experimental Biology and Medicine, at Lexington, Kentucky, 1960.

ol. 7
961

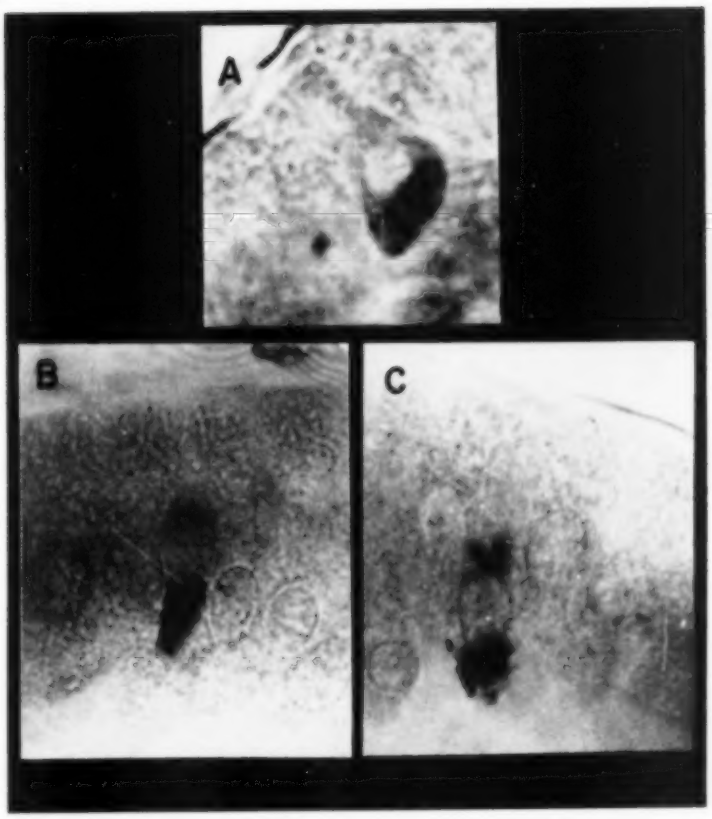


FIG. 1. Supravital preparations of normal rabbit enterochromaffin cells. A. Characteristic flask shape. × 454. B, C. Characteristic distribution of granules. × 360.

facing page 96

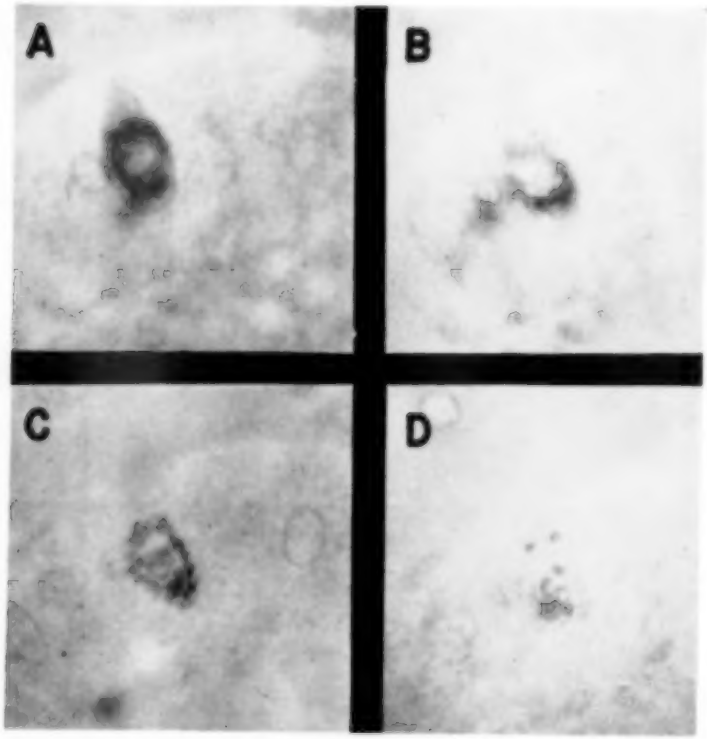


FIG. 2. Supravital preparation of a rabbit enterochromaffin cell after administration of 5 mg of reserpine per kg. A, 3 hr. B, 4 hr. C, 5 hr. D, 6 hr. × 873.

it off, using as a pusher the narrow side of a glass microscope slide, pressed firmly against the tissue at an angle of about 30°. Often, the mucosa could be removed in layers, the first by a gentle scraping, the second by a more forceful one. Under these conditions there were usually more enterochromaffin cells found in the second layer than in the first, in conformity with greater occurrence of these cells in the deeper portions of the mucosal folds noted in our silver-stained preparations.[2]

Small masses of the duodenal mucosa scrapings were transferred to watch glasses and covered with a 1% solution of neutral red in 0·9% saline. These were rotated for from 30 to 60 sec, then the excess dye was poured off and the stained tissue in the watch glass was rinsed with several changes of Hank's balanced salt solution,[5] buffered at pH 7·5, until no more dye was freely removed from the tissue. With dissecting needles, a small mass of this stained tissue, approximately 1 mm^3, was transferred to a drop of buffered balanced salt solution in the center of a circle of petrolatum on a glass microscope slide. The tissue was gently teased apart with the needles and attempts were made to orient the tissue masses into sheets or strands. A cover slip was then placed on top of the preparation and sealed to the circle of petrolatum, care being taken to determine that the seal was intact completely around the circumference of the tissue spread.

Microscopic examination at magnification × 100 revealed enterochromaffin cells in the mucosal scrapings. Individual cells were followed for prolonged periods at this and at higher magnifications. It was possible to examine these cells under phase contrast as well as bright field optical conditions. Photographs of these cells at appropriate intervals recorded the changes in them produced by the actions of drugs.

For comparison with the above supravital staining method, preparation of microscopic sections and staining of the enterochromaffin cells was performed according to methods reported previously.[2] Estimation of the magnitude of granulation in stained sections was performed by counting and weighting the cells in ten high-power fields in each of four sections and calculating a weighted granulation index according to the Hartrofts.[6] The significance of the difference between the means of the weighted indices of the experimental and corresponding control sections was determined by the *t*-test.

RESULTS

In scrapings of duodenal mucosa stained with neutral red, one may observe microscopically a small area of tissue with its component cells arranged as they occur normally *in vivo*. Two kinds of cells in these preparations contain granules stained with neutral red. One of them is not closely associated with the epithelial cells of the mucous membrane, is round, and its granules are extremely dense and so densely packed as to obscure the nucleus of the cell. These we identify as mast cells; although not very numerous in the rabbit duodenum, they do occur regularly.

The other cell, with granules stained red under these conditions, contains fewer and smaller granules than the mast cell, and the granules are predominantly in one end of the cell, the nucleus being seen near the middle. When one examines a piece of tissue properly oriented, these cells are seen to be between the epithelial cells of the mucosa, and in the same layer with them. A typical cell of this species is flask-shaped, with the wide end, containing the granules, toward the basement membrane of the epithelium. The narrow neck of the cell, free of granules, extends to the striated

border of the epithelium. The nucleus is characteristically seen at the shoulder of this cell (Fig. 1). These cells appear to fit the requirements for identification as enterochromaffin cells. Particularly, they are consistent with the electron microscopic observations of Taylor and Hayes[7] on the structure of this cell.

As a test for this method of examining enterochromaffin cells, we can study them under conditions which are thought to cause degranulation, as revealed by the older methods of examining stained sections. One such condition is the administration of reserpine, 5 mg/kg.[2] A series of photographs of one enterochromaffin cell from the duodenum of a reserpinized rabbit is shown in Fig. 2. It can be seen that loss of vital

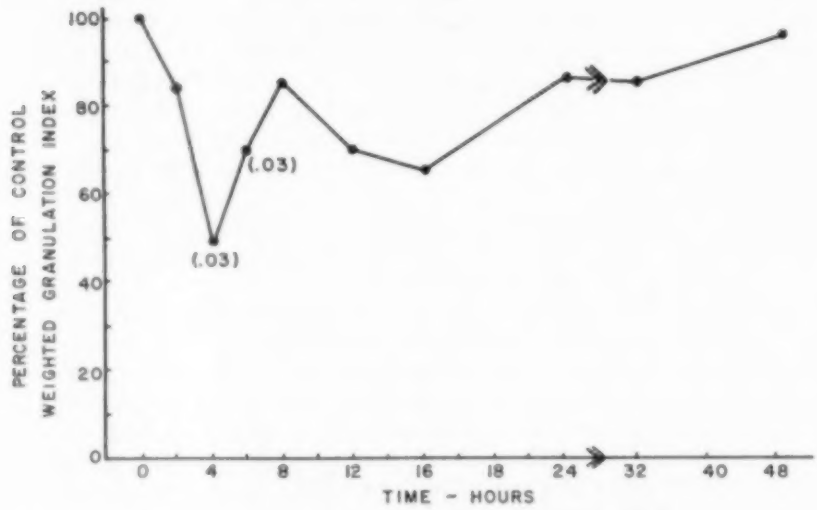


FIG. 3. Change in weighted granulation indices of rabbit duodenum sections after the intravenous administration of 2 mg of colchicine per kg. Figures in parentheses are the *P*-values for observations that show significant differences from the controls.

staining of this cell began in from 3 to 4 hr and was virtually complete in 6 hr. With saline controls, no changes such as those recorded here were seen in this period of time. Although some enterochromaffin cells in each preparation were not degranulated, the vast majority showed a loss of granule-staining in response to reserpine. Mast cells were still recognizable at a time when the specific destaining of enterochromaffin cells had already occurred.

As a further test of this method of examining enterochromaffin cells, we studied them both supravitally and in stained sections following the administration of colchicine. Fig. 3 gives the results of the quantitative evaluation of cellular granulation in rabbits sacrificed at various time intervals following the intravenous administration of 2 mg of colchicine per kg. Significant degranulation ($P = 0{\cdot}03$) occurred at 4 and 6 hr after administration; however, the difference between the treated and control animals had essentially disappeared after 24 hr. Likewise, in supravital preparations of duodenal mucosa made by the neutral red method 2 hr after the

Vol. 7
1961

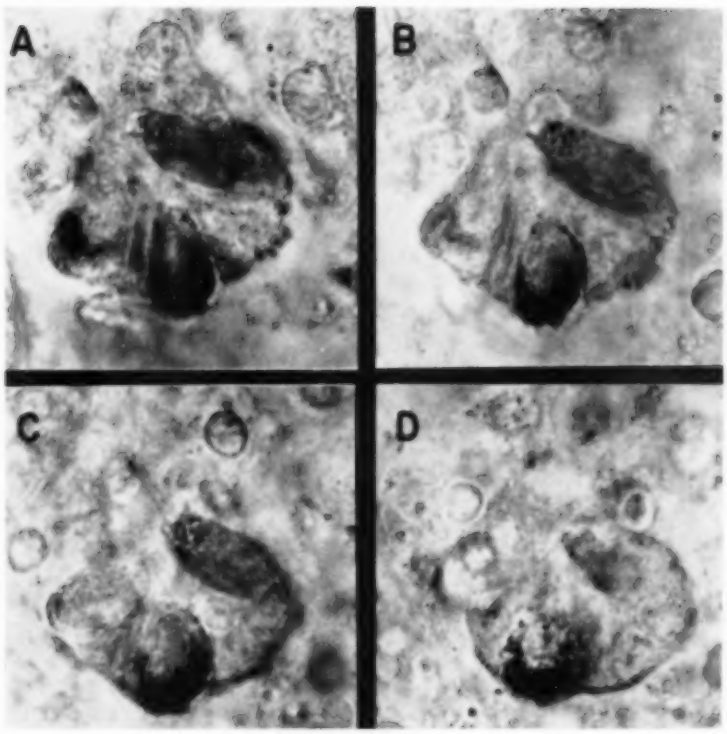


FIG. 4. Supravital preparation of a rabbit enterochromaffin cell after the intravenous administration of 2 mg of colchicine per kg: A, 3 hr; B, 4 hr; C, 5 hr; D, 6 hr. × 360.

facing page 98

BLANK
PAGE

Vol. 7
1961

intravenous administration of 2 mg of colchicine per kg, evidence of cellular change was observed in a number of instances in the succeeding few hours (Fig. 4).

DISCUSSION

The use of supravital-stained preparations of duodenal mucosa directly demonstrates loss of staining of enterochromaffin cell granules in response to reserpine. The delay required for this occurrence and the delay generally observed in producing hyperperistalsis are parallel and indicate the functional role of released serotonin in producing this response.

Though one cannot readily obtain quantitative data from the examination of a few single cells, such direct observation is a rapid method for screening drugs for possible effects on cells of this type.

Colchicine (or colchicum) has been known since antiquity to produce a characteristic delayed hyperperistalsis when administered in large doses. Fuehner and Rehbein[8] showed long ago that this drug had no direct action on strips of intestinal muscle. Observations reported here indicate that, like reserpine, colchicine may induce a delayed release of the contents of enterochromaffin granules. This is shown both by the study of fixed and stained sections and by the examination of supravital-stained enterochromaffin cells. This finding indicates that serotonin may play a functional role in producing the hyperperistalsis associated with colchicine administration.

SUMMARY

Supravital staining with neutral red is shown to distinguish enterchromaffin cells. These cells show a destaining response to reserpine. Both in stained sections and by supravital staining, colchicine is also shown to induce delayed loss of granulation of enterochromaffin cells.

REFERENCES

1. E. Bulbring and L. Crema, *J. Physiol.* **146**, 29 (1959).
2. B. H. Marks, R. W. Sorgen and E. R. Hayes, *Proc. Soc. Exp. Biol., N.Y.* **97**, 413 (1958).
3. E. Bulbring and R. C. Y. Lin, *J. Physiol.* **140**, 381 (1958).
4. E. P. Benditt and R. L. Wong, *J. Exp. Med.* **105**, 509 (1957).
5. J. H. Hanks, *J. Cell. Comp. Physiol.* **31**, 235 (1948).
6. P. M. Hartroft and W. S. Hartroft, *J. Exp. Med.* **97**, 145 (1953).
7. J. Taylor and E. R. Hayes, Reported to meeting of Electron Microscope Society of America, Columbus, Ohio, Sept. (1959).
8. H. Fuehner and M. Rehbein, *Arch. exp. Path. Pharmak.* **79**, 1 (1916).

Biochemical Pharmacology, 1961, Vol. 7, pp. 100–108. Pergamon Press Ltd., Printed in Great Britain.

SYNTHESIS OF 4-THIAMETHIONINE AND ITS EFFECT ON ENERGY METABOLISM AND AMINO ACID INCORPORATION INTO PROTEIN OF EHRLICH ASCITES TUMOUR CELLS

M. RABINOVITZ and J. M. JOHNSON

National Cancer Institute, National Institutes of Health, Bethesda, Md.

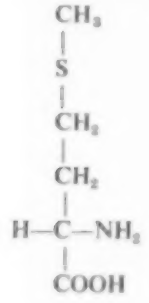
(*Received* 28 *November* 1960)

Abstract—The methionine analog, 4-thiamethionine, was synthesized and found to be an inhibitor of incorporation of several amino acids into Ehrlich ascites protein *in vitro*. The inhibition was not prevented by methionine. Glutathione prevented the inhibition but could not bring about a reversal once the block was initiated. Glucose both prevented and reversed the inhibition, thus indicating that 4-thiamethionine interfered with the oxidative energy supply supporting protein synthesis.

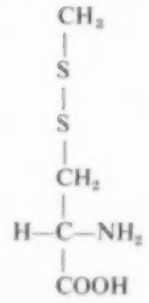
INTRODUCTION

THE possibility of introducing a permanent lesion within the protein synthetic mechanism of cells through the use of amino acids bearing highly reactive functional groups has received attention in various laboratories.[1–3] Since there is inherent in the process of protein synthesis a marked specificity with regard to the structure and configuration of amino acids utilized,[4] such inhibitors should bear a close structural resemblance to those found in protein.

One alteration in the structure of a natural amino acid which has produced an effective antagonist is the substitution of sulfur for the fourth carbon of lysine, to form S(β:aminoethyl) cysteine or 4-thialysine. This analog is a potent antimetabolite of lysine in bone marrow cells[5] and also in the Ehrlich ascites tumor.* Accordingly, we have synthesized the corresponding analog of methionine, 4-thiamethionine, which is the mixed disulfide of methyl mercaptan and cysteine.



Methionine



4-Thiamethionine

* M. Rabinovitz and J. M. Johnson, unpublished observations.

This analog, which bears the reactive disulfide function, might be expected to participate in disulfide interchange[6] with sulfhydryl groups at potential sites specific to methionine at intermediate stages in protein synthesis. A study of the characteristics of its inhibition of protein synthesis, however, indicated that the analog did not function as a methionine antagonist, but reduced the oxidative energy supply availfor protein synthesis.

MATERIALS AND METHODS

Synthesis of 4-*thiamethionine*

The compound was prepared by the dismutation between dimethyl disulfide and cysteine, a method previously reported to be applicable for the general synthesis of unsymmetrical disulfides.[7]

Lithium hydroxide monohydrate, 1·85 g (0·045 moles), in 21 ml of oxygen-free water, was slowly added over a period of 20 min to a stirred refluxing solution of L-cysteine hydrochloride monohydrate, 7·0 g (0·04 moles), and dimethyl disulfide, 12 g (0·128 moles), in methanol (50 ml). The solution became turbid upon addition of a few milliliters of alkali and a heavy precipitate had formed by the time all the lithium hydroxide was added. The reaction mixture was cooled and the precipitate was filtered under suction, washed with ethanol and ether and dried. The yield of crude product was 4·8 g. A small sample was dissolved in water and analysed by descending paper chromatography with butanol–water–acetic acid (10 : 5 : 2).* The principal ninhydrin-reactive spot had an R_f of 0·49; L-methionine gave an R_f of 0·48. The compound gave the typical violet coloration with ninhydrin. A cystine contaminant was also recognized, which amounted to 20 per cent of the reaction product.

The crude product was purified by removing the cystine at the pH of its minimum solubility.[8] It was suspended in 200 ml of water and brought into solution by dropwise addition of hydrobromic acid. Cystine was precipitated by adjusting the solution to pH 3 with lithium hydroxide. The pH of the filtrate was raised to pH 6·7, acetone was added and the product was obtained as white microcrystals. It was filtered under suction and washed with ethanol and acetone.

Paper chromatography indicated that the pH 3-precipitate contained almost all of the cystine, together with much of the product. No attempts were made to recover additional material from the pH 3 precipitate. The product, 4-thiamethionine, (1·2 g), contained only a trace level of cystine, as indicated by paper chromatography, and was used in all subsequent studies. It melted with decomposition at 209–210 °C (corr.), in a sealed tube. *Anal.*, Calc. for $C_4H_9O_2NS_2$: C, 28·72; H, 5·42; N, 8·38; S, 38·34. Found: C, 28·78; H, 5·52; N, 8·37; S, 38·09. Although L-cysteine was used as a starting material, some racemization may have occurred under the alkaline conditions of the reaction.

Metabolic studies

Ehrlich ascites cells were obtained from C_3H mice which had been inoculated 1 week previously. The animals were sacrificed by cervical dislocation, and the ascitic fluid was drained through an abdominal incision into beakers placed in ice. The cells were then washed by suspension and sedimentation in ice-cold Krebs–Ringer-phosphate (KRP) buffer containing 0·12 M sodium chloride, $1·3 \times 10^{-2}$ M potassium chloride,

* All paper chromatography reported in this article was performed under these conditions.

$1{\cdot}8 \times 10^{-3}$ M calcium chloride, $6{\cdot}5 \times 10^{-4}$ M magnesium sulfate, and 0·01 M sodium phosphate, pH 7·2. The cells were then suspended in two volumes of buffer and filtered through glass wool to remove small clots. The radioactive amino acids used were DL-methionine-2-C^{14} and the uniformly labeled, natural isomers of isoleucine, leucine, lysine, phenylalanine and valine. These were mixed with corresponding non-radioactive material to make solutions of appropriate concentration and specific radioactivity.

The main compartment of Warburg flasks contained radioactive amino acid, a freshly prepared solution of 4-thiamethionine or other additives in buffer in a volume of 1·5 ml; the sidearms contained 0·5 ml of the Ehrlich tumor cell suspension. After temperature equilibration at 37·5 °C for 7 min, the cells were tipped into the main compartment and incubated for various periods. At times alkali and filter paper strips were added to the center well and oxygen consumption was determined with air as the gas phase.

Anaerobic glycolysis was studied with 95% nitrogen, 5% carbon dioxide as the gas phase, in buffer containing sodium chloride, 0·10 M; potassium chloride, $1{\cdot}3 \times 10^{-2}$ M; calcium chloride, $1{\cdot}8 \times 10^{-3}$ M; magnesium sulfate $6{\cdot}5 \times 10^{-4}$ M; glucose $1{\cdot}5 \times 10^{-2}$ M; sodium bicarbonate, 2×10^{-2} M and sodium phosphate 5×10^{-3} M, pH 7·4. When glucose was added as substrate under aerobic conditions, the above buffer with a gas phase of 95% oxygen and 5% carbon dioxide was used.

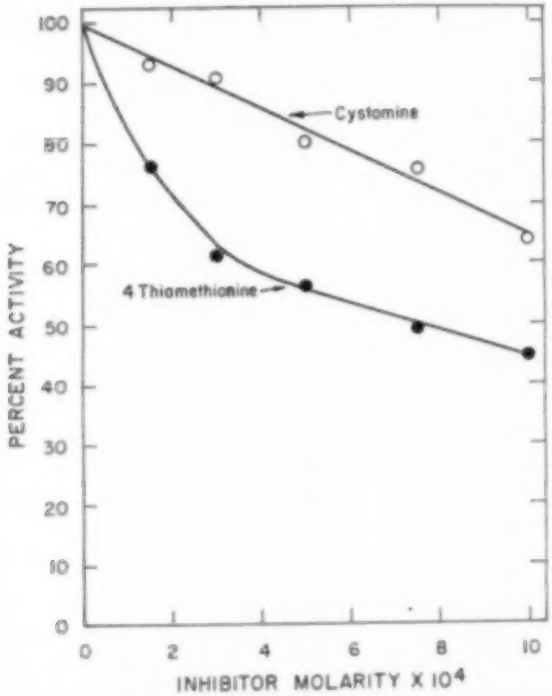


FIG. 1. The inhibition by several concentrations of 4-thiamethionine and cystamine of lysine incorporation into Ehrlich ascites cell protein. The uninhibited control incorporated $1{\cdot}38 \pm 0{\cdot}01$ μmoles lysine per g protein during the 15-min incubation period. The L-lysine concentration was 5×10^{-4}M.

At the end of the incubation period the reaction was terminated by the addition of an equal volume of 20% trichloroacetic acid (TCA). Precipitated material was washed three times with 5% TCA, once with 5% TCA at 90 °C for 15 min, once with 5% TCA at room temperature, once with absolute ethanol, three times with 3 : 1 ethanol : ether mixture at 62 °C for 5 min, and twice with absolute ether. The protein was plated on aluminum discs and counted in a flow-gas counter.

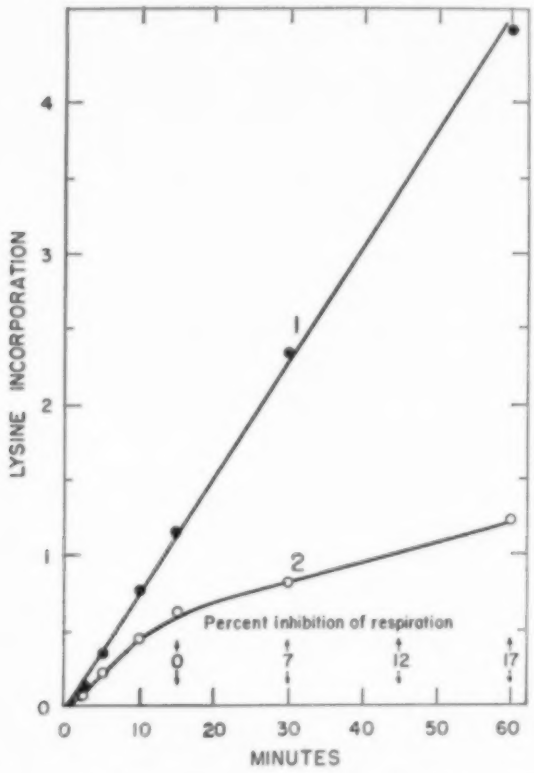


FIG. 2. Progressive increase in inhibition of lysine incorporation by 4-thiamethionine: (1) uninhibited control; (2) inhibition by 5×10^{-4} M 4-thiamethionine. The L-lysine concentration was 5×10^{-4} M. The per cent inhibition of respiration is indicated for four time intervals. Incorporation is expressed as μmoles lysine incorporated per g of protein.

RESULTS

General characteristics of the inhibition

4-Thiamethionine inhibited the incorporation of all the amino acids tested into proteins of the Ehrlich ascites cell. The effect of several concentrations of the inhibitor on lysine incorporation during a 15-min incubation period is shown in Fig. 1. Higher concentrations could produce complete inhibition during this period. The lower concentrations, however, are more effective during longer incubation periods (Fig. 2). This phenomenon is similar to that previously observed with dinitrophenol.[9] The inhibition is not due to an interference in the passage of radioactive amino acid into

the cell, since the incorporation of intracellular isoleucine was inhibited to an equal extent (Table 1).

A strong odor of methyl mercaptan was recognized after incubation of cells with the inhibitor. As shown in Fig. 1, another disulfide, cystamine, was a less effective inhibitor at equivalent concentrations. Dimethyl disulfide was not an inhibitor.

TABLE 1. INHIBITION BY 4-THIAMETHIONINE AND CYSTAMINE OF INCORPORATION OF INTRACELLULAR *iso*LEUCINE INTO PROTEIN OF THE EHRLICH ASCITES CELL

	Increase in protein specific activity after second incubation (Counts/min per mg protein)	Inhibition (per cent)
Uninhibited control	394 ± 1*	—
4-Thiamethionine, 5×10^{-4} M	193 ± 6	51
Cystamine, $1{\cdot}5 \times 10^{-3}$ M	210 ± 1	47

The cells were isolated from ascitic fluid and washed once with KRP buffer. Two milliliters of cells were then supplied with an intracellular pool of radioactive isoleucine by incubating them for 2 min with 4·8 μmoles L-*iso*leucine-U-C^{14}, assaying $1{\cdot}15 \times 10^6$ counts/min in a total volume of 8·4 ml. The cells and medium were cooled to 0 °C in ice, the medium was removed by centrifugation and the cells washed twice with cold KRP buffer to remove all remaining extracellular *iso*leucine-U-C^{14}. They were then resuspended in buffer for a second incubation under standard conditions.

* Deviation from the mean of duplicate incubations.

Prevention of the inhibition

The inhibition of methionine incorporation by 4-thiamethionine was non-competitive at several inhibitor concentrations. This is illustrated for two inhibitor concentrations in Fig. 3. Also, as shown in Table 2, methionine did not prevent the inhibition of incorporation of other amino acids.

Reduced glutatione prevented the action of 4-thiamethionine (Table 3) by reacting with the inhibitor,* but it could not effect a reversal after the inhibition had been initiated.

Inhibition in relation to energy source

Higher concentrations of 4-thiamethionine caused a progressively increasing inhibition of respiration of the Ehrlich ascites cells. However, at a concentration of 5×10^{-4} M, its effect on oxygen uptake became apparent only after inhibition of protein synthesis was complete (Fig. 2).

Under conditions of anaerobic glycolysis, 4-thiamethionine had comparatively little effect on lysine incorporation (Fig. 4) in a concentration at which it significantly inhibited incorporation supported by respiration. The analog stimulated glycolysis by 20 per cent when present in the medium at this concentration (5×10^{-4} M). A similar stimulation of anaerobic glycolysis by dinitrocresol[10] and dinitrophenol[11] has been reported. Furthermore, the inhibitor was ineffective aerobically in the presence of glucose (Table 4), which can also reactivate the completely inhibited system (Fig. 5). Under similar conditions no reactivation could be achieved by the addition of reduced glutathione, 0·01 M, or cysteamine, 0·005 M.

* This reaction can be followed by paper chromatography of the 4-thiamethionine–glutathione mixture.

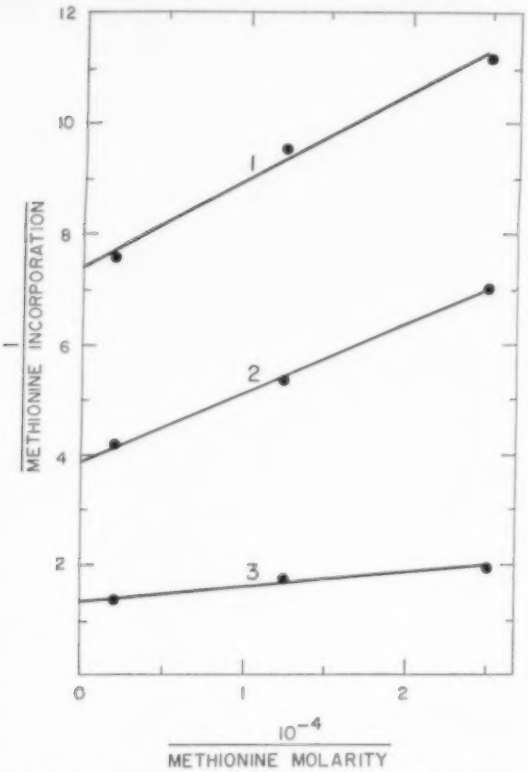


FIG. 3. Double reciprocal plot showing non-competitive inhibition of methionine incorporation into Ehrlich ascites cell protein by two concentrations of 4-thiamethionine: (1) inhibition by 5×10^{-3} M 4-thiamethionine; (2) inhibition by 5×10^{-4} M 4-thiamethionine; (3) uninhibited control. Incorporation is expressed as μmoles methionine incorporated per g of protein during the 15-min incubation period.

TABLE 2. 4-THIAMETHIONINE INHIBITION OF PHENYLALANINE AND *iso*LEUCINE INCORPORATION INTO EHRLICH ASCITES CELL PROTEIN, AND ITS INDEPENDENCE OF METHIONINE CONCENTRATION

Amino acid incorporated	4-Thiamethionine concentration	Inhibition			
		No methionine added	L-methionine / 4-thiamethionine (molar ratio)		
			5	10	25
	(M)	(% of uninhibited control)			
L-Phenylalanine	5×10^{-4}	66	—	66	51
L-*iso*Leucine	3×10^{-4}	43	46	57	—

The L-phenylalanine and L-*iso*leucine were added to give a final concentration of 5×10^{-4} M.

TABLE 3. PREVENTION OF 4-THIAMETHIONINE INHIBITION OF LYSINE INCORPORATION BY REDUCED GLUTATHIONE

Lysine incorporation with			
Buffer only	4-Thiamethionine 5×10^{-4} M	Glutathione 5×10^{-3} M	4-Thiamethionine 5×10^{-4} M and glutathione 5×10^{-3} M
(μmoles/g protein per 15 min incubation period)			
1·80 ± 0·01*	0·96 ± 0·00	1·83 ± 0·01	1·70 ± 0·03

* Deviation from the mean of duplicate incubations. The L-lysine concentration was 5×10^{-4} M.

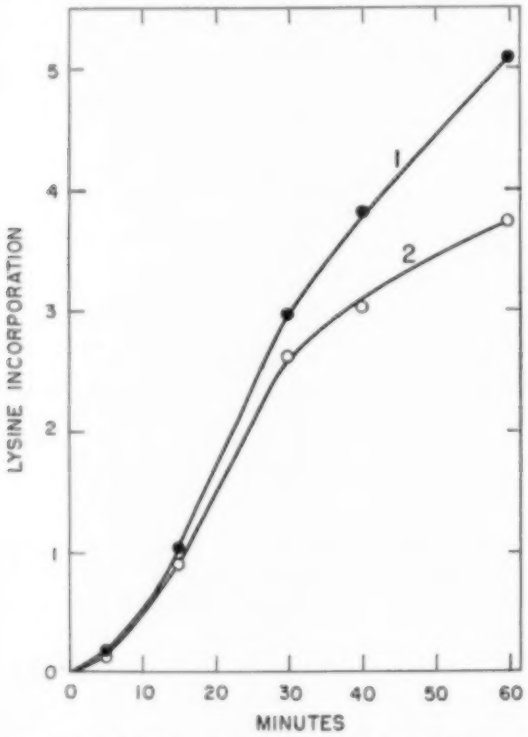


FIG. 4. Inhibition by 4-thiamethionine of lysine incorporation under conditions of anaerobic glycolysis: (1) uninhibited control; (2) inhibition by 5×10^{-4} M 4-thiamethionine. The L-lysine concentration was 5×10^{-4} M. Anaerobic glycolysis was stimulated by 20 per cent in the presence of 4-thiamethionine. Incorporation is expressed as in Fig. 2.

The results are in accord with the view that the analog or its metabolic products interfere with amino acid incorporation of respiring tumor cells by reducing the energy supply made available through oxidative phosphorylation, as do dinitrophenol[9] and other compounds.[12]

Vol. 7
1961

TABLE 4. PREVENTION BY GLUCOSE OF 4-THIAMETHIONINE INHIBITION OF LYSINE AND VALINE INCORPORATION UNDER AEROBIC CONDITIONS

C^{14}-Amino acid 5×10^{-4} M	Incorporation with			
	Buffer only	4-Thiamethionine 5×10^{-4} M	Glucose $1{\cdot}5 \times 10^{-2}$ M	4-Thiamethionine 5×10^{-4} M and glucose $1{\cdot}5 \times 10^{-2}$ M
	(μmoles/g protein per 15 min incubation period)			
L-Lysine	1·66	0·83	1·68	1·50
L-Valine	1·72	0·75	1·69	1·47

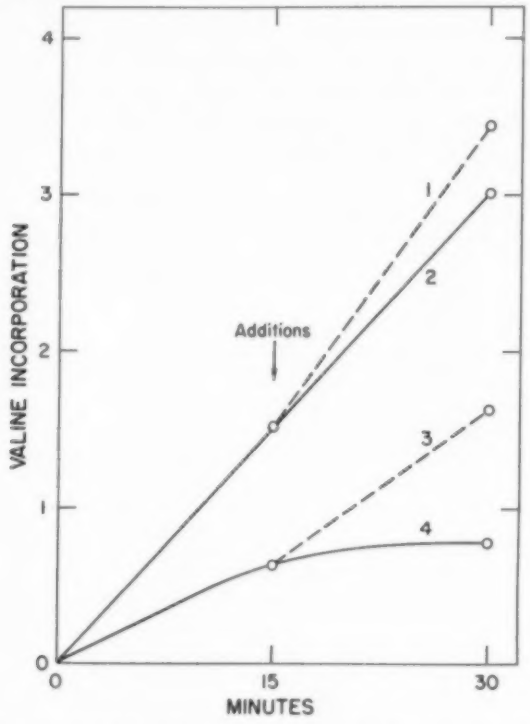


FIG. 5. Relief by glucose of 4-thiamethionine inhibition of valine incorporation: (1) uninhibited control; glucose, 0·015 M final concentration, added at 15-min interval; (2) uninhibited control; buffer only added at 15-min interval; (3) inhibition by 5×10^{-4} M 4-thiamethionine; glucose, 0·015 M final concentration, added at 15-min interval; (4) inhibition by 5×10^{-4} M 4-thiamethionine; buffer only added at 15-min interval. Incorporation is expressed as μmoles valine incorporated per g protein. The L-valine concentration was 5×10^{-4} M.

DISCUSSION

The results indicate that 4-thiamethionine interferes primarily with the energy supply of the cells made available by respiration, and not with the protein synthetic mechanism of the cell. The ability of glucose to relieve, as well as to prevent, the

inhibition indicates that this substrate acts by furnishing the cells with an alternate energy source through glycolysis. Quastel and Bickis[12] have suggested that tumor cells may be resistant to a variety of inhibitors through their ability to utilize alternately either oxidative or glycolytic energy sources. It is of interest, also, that glutathione and cysteamine could not reverse the inhibition, although such reversal can be demonstrated in disulfide-inhibition of individual enzymes.[13]

The mechanism for protein synthesis is capable of introducing each amino acid, many close analogs of one another, into the proper sequence of individual proteins. Recent studies have indicated that specificity is shown in reactions that may represent early stages of protein biosynthesis, such as amino acid activation and transfer to soluble ribonucleic acid.[14] These considerations would appear to run counter to the view[15] that effective analogs of amino acids may deviate considerably in structure from the natural metabolite. Although this view is valid for other systems[16] the high specificity required for competitive amino acid antagonism to protein synthesis would indicate that this concept is not applicable here. The alternative, that protein synthesis may be blocked* by an amino acid bearing a highly reactive functional group which is also a close structural analog of a natural amino acid, imposes severe restrictions on the number of potential compounds which could be designed.

* Studies with labeled amino acids and their antagonists indicate that most amino acid antagonists do not inhibit the protein synthetic mechanism, but to some extent replace the corresponding metabolite during the synthesis of protein.[4] The resultant formation of unnatural proteins and enzymes within the cell possibly leads to inhibition of growth and decrease in the associated net increase in protein. A direct reversible block of globin synthesis at the microsomal site has been reported.[17]

Vol. 7
1961

REFERENCES

1. F. BERGEL and J. A. STOCK, *J. Chem. Soc.* 2409 (1954).
2. J. M. RAVEL, T. J. MCCORD, C. G. SKINNER and W. SHIVE, *J. Biol. Chem.* **232**, 159 (1958).
3. B. R. BAKER, *Cancer Chemotherapy Rep.* **4**, 1 (1959).
4. M. VAUGHAN and D. STEINBERG, *Advanc. Protein Chem.* **14**, 115 (1959).
5. M. RABINOVITZ and R. K. TUVE, *Proc. Soc. Exp. Biol., N.Y.* **100**, 222 (1959).
6. E. V. JENSEN, *Science* **130**, 1319 (1959).
7. D. T. MCALLAN, T. V. CULLUM, R. A. DEAN and F. A. FIDLER, *J. Amer. Chem. Soc.* **73**, 3627 (1951).
8. A. R. T. MERRILL, *J. Amer. Chem. Soc.* **43**, 2688 (1921).
9. M. RABINOVITZ, M. E. OLSON and D. M. GREENBERG, *J. Biol. Chem.* **213**, 1 (1955).
10. G. H. A. CLOWES and A. K. KELTCH, *Proc. Soc. Exp. Biol. ,N.Y.* **86**, 629 (1954).
11. L. SLECHTA and R. BENTLEY, *Arch. Biochem. Biophys.* **89**, 41 (1960).
12. J. H. QUASTEL and I. J. BICKIS, *Nature, Lond.* **183**, 281 (1959).
13. J. B. WALKER and M. S. WALKER, *Arch. Biochem. Biophys.* **86**, 80 (1960).
14. M. B. HOAGLAND, *The Nucleic Acids* (Edited by E. CHARGAFF and J. N .DAVIDSON) Vol. III, pp. 383–4. Academic Press, New York (1960).
15. H. F. GRAM, C. W. MOSHER and B. R. BAKER, *J. Amer. Chem. Soc.* **81**, 3103 (1959).
16. H. G. MAUTNER, *Yale J. Biol. Med.* **32**, 323 (1960).
17. M. RABINOVITZ and H. MCGRATH, *J. Biol. Chem.* **234**, 2091 (1959).

Biochemical Pharmacology, 1961, Vol. 7, pp. 109–122. Pergamon Press Ltd., Printed in Great Britain.

AN ABNORMAL SERUM COMPONENT IN EXPERIMENTAL POISONING BY CADMIUM AND OTHER METALS

D. J. LAWFORD*

Department for Research in Industrial Medicine
(Medical Research Council), The London Hospital, London, E.1

(*Received* 22 *December* 1960)

Abstract—An abnormal serum component was observed 24 hr after administration of a single dose of cadmium to albino rats by intraperitoneal injection, which gradually disappeared from the serum over a period of 9 days. The component was not observed on starch gel electrophoresis following very high or very low dosage, the optimum level being found to be 1 mg/kg of cadmium. The rate of migration of the material was not affected by the addition of CaEDTA to the system or the use of a more acid buffer.

Ammonium sulphate fractionation showed that the abnormal serum component was precipitated at between 35–40% saturation.

The *in vitro* addition of cadmium to samples of serum in varying concentrations revealed progressive alteration in the migration rate of the albumin band, but indicated no line comparable with the abnormal serum component.

The production of varying amounts of the component was observed following the injection of other metals, and mercury appeared to give a stronger line than cadmium, whereas lead gave no band at all.

The component was isolated from cadmium-poisoned rat serum in a reasonable state of purity, by means of neutral salt fractionation and zone electrophoresis.

An antiserum to the purified material was prepared in rabbits, and after suitable treatment, this antiserum was used to show the presence of the abnormal component in rat serum following very low intraperitoneal doses of cadmium.

INTRODUCTION

THE ingestion of small amounts of soluble cadmium salts has been shown to produce a rapid and severe anaemia[1–5] and an apparent considerable increase in the serum transferrin content of young albino rats,[6] whereas the injection of soluble cadmium salts can produce certain changes in the normal plasma protein relationship. Truhaut and Boudene,[7] in a study of the distribution of cadmium in rats following acute, subacute, and chronic administration by injection, found that following chronic administration, there appeared to be progressive hypoalbuminaemia and hyperglobulinaemia (an initial increase and a subsequent decrease in the α-globulins and a steady rise in the β-globulins levels). Barni *et al.*,[8] working along the same lines, found that the continuous injection of high doses of cadmium to rabbits produced an increase in the total blood proteins, a reduction in the albumin and α_1-globulin content being more than compensated by a progressive increase in the α_2, β and γ-globulins. As the poisoning progressed, it was observed that the α-lipoproteins decreased, while

Abbreviations: A.S.C., abnormal serum component. Part of work approved for Ph.D. thesis (London).

* Present address: Abbott Laboratories Ltd., 8 Baker Street, London, W.1.

the α_2, β and γ-glycoproteins increased. Odescalchi *et al.*[9] reported similar findings, and found in addition, an inverted albumin–globulin ratio, which suggested to them that the modifications in the concentrations of the serum proteins may have been related to certain liver changes that they had observed.

Stokinger and Wagner[10] have reported that the α-globulin fractions of the sera of dogs and rabbits were elevated, following exposure to small doses of cobalt.

The aim of the present work was to investigate in greater detail the serum protein changes produced in the albino rat, following the parenteral administration of cadmium salts.

MATERIALS AND METHODS

Animals

Adult Chester–Beatty strain rats were used.

Starch

Specially prepared Connaught Research Laboratories starch was used.

Buffer solutions

Borate buffer solutions. This solution was prepared in batches of 2 l. at a time and consisted of the following composition: 37·104 g of AnalaR boric acid, 4·8 g of AnalaR sodium hydroxide, made up to 2 l. with distilled water.

TRIS buffer solution. This solution was prepared in batches of 2 l. at a time consisting of the following composition: 18·413 g of tris-(hydroxymethyl)-aminomethane (melting point 170°C), 2·102 g of AnalaR citric acid, made up to 2 l. with distilled water.

Staining solution

A saturated solution of the dyestuff naphthalene black 10B 200 in the following solution: methanol, five parts; glacial acetic acid, one part; water, five parts.

Washing solution

The staining solution, minus the dyestuff.

Buffer systems

Continuous method. In this method, the Tris buffer solution was used to make up the gel and was also used in the buffer reservoir boxes. The system was run at 520 V, 10 mA and a voltage gradient of 2 V/cm for 16 hr.

Discontinuous method.[11] The gel was made up using Tris buffer solution, the reservoir boxes being filled with borate buffer solution. The system was run at 520V, 20 mA and a voltage gradient of 6 V/cm for 4 hr.

Starch gel electrophoresis

The method of Smithies[12] was used throughout these experiments.

Preparative electrophoresis

The apparatus designed by Porath[13] was used, in conjunction with the procedure of Gedin and Porath.[14]

Vol. 7
1961

Paper electrophoresis

The method of Kunkel and Tiselius[15] was used.

Apparatus for immune reactions

For the immune reactions in tubes, the method of Oakley and Fulthorpe[16] was used. In addition, the techniques of Öuchterlony,[17] and the micro-modification of Öuchterlony and Wadsworth[18] were employed.

Immunoelectrophoresis

It was found that starch was the most satisfactory medium for the separation of the abnormal serum component in rat serum, and the following method was used for the immunoelectrophoretic studies.

A plastic dish, 11·5 cm by 17·5 cm was filled to a depth of 2–3 mm with a solution of 1% agar (Ionagar no. 2 Oxoid) in sterile Tris buffer containing 0·5% sodium azide to prevent bacterial growth. The serum to be examined was subjected to starch gel electrophoresis in the usual manner, using the continuous buffer system, and slices were cut and placed face downwards on the surface of the agar. Strips of Whatman 3MM filter paper, 0·3 mm wide and of similar length to the starch gel slices were were dipped in antiserum and placed on the agar surface, parallel to the starch slices and at a distance of 3–4 mm from them (Fig. 1). The dish was carefully sealed and incubated at 37 °C for 7 days.

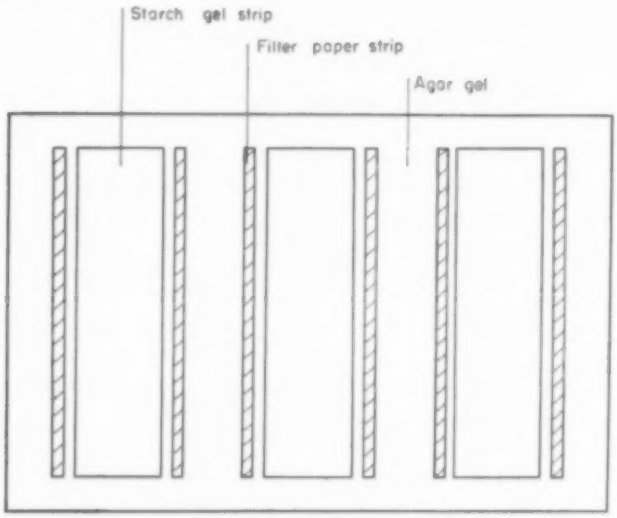


FIG. 1. Apparatus for immunoelectrophoresis. Actual size.

After marking the positions occupied by the starch gels and the filter paper strips, these were removed and the agar gel was suspended in trays of saline, with frequent changes for 3 days, in order to remove remaining soluble material. The agar gel was finally placed on a glass plate, covered with a fitted sheet of thick filter paper and allowed to dry. When dry, the surface was rinsed with water to remove the filter paper, and finally stained in methanol–water–acetic acid solution saturated with

naphthalene black, as described earlier. After suitable rinsing, the gel was dried and photographed.

Serum changes following parenteral administration of cadmium

1. *Appearance of an abnormal serum component.* Two groups of five male Chester–Beatty rats, aged 3 months, and weighing approximately 300 g were taken, one group serving as controls, the second group being given 1 mg/kg of inactive cadmium (as cadmium chloride) by intraperitoneal injection. Samples of blood were taken from each animal by cardiopuncture after 24 hr. The blood was allowed to clot and the sera were submitted to starch gel electrophoresis using both the continuous and the discontinuous buffer systems.

Although several changes of a minor character were observed between the control and experimental animals, the most outstanding feature was the appearance of a new band in the sera of the cadmium rats, which was not apparent in the sera of the control rats (Fig. 2). This band could be readily observed when the continuous buffer system was used, but was slightly less obvious using the discontinuous buffer system.

2. *Effect of varying doses of cadmium.* Five male adult rats were given 0, 0·025, 0·1, 0·2 and 1 mg/kg body weight of inactive cadmium as cadmium chloride, daily for 4 days. Starch gel analyses were made on sera from these animals taken 1, 2, 3 and 4 days after the commencement of dosing. The A.S.C. band appeared after 24 hr and increased to a maximum up to 3 days, but appeared to be slightly weaker after a further day (Fig. 3). Other changes were seen to occur at the 1 mg/kg level, notably a reduction of the albumin band, and a visible decrease in the globulin level similar to those observed when cadmium was administered orally.

Properties of the abnormal serum component

1. *Effect of pH on electrophoretic mobility.* Serum containing the A.S.C. was subjected to starch gel electrophoresis using a continuous Tris buffer system adjusted to pH 7·0. No alteration in the speed of migration of the A.S.C. was seen, although other serum components migrated a smaller distance in a measured time.

2. *Effect of incorporating EDTA.* The addition of a small amount of disodium dihydrogen ethylenediaminetetra-acetic acid to the serum, made no difference to the speed of migration of the A.S.C., suggesting that it was not cadmium-bearing, a finding which was subsequently confirmed.

3. *Rate of appearance and disappearance.* Three male rats were given a single dose of 1 mg/kg of cadmium, as inactive cadmium chloride, intraperitoneally. Blood samples were collected from the tail at 0, 6 and 24 hr after the injections, and thereafter daily for 2 weeks. Starch gel electrophoresis using the continuous buffer system showed that the A.S.C. band first appeared after 24 hr, reached a maximum after 2 days, and gradually disappeared with time, becoming invisible 9 days after the injection.

4. *Optimum dose of cadmium to produce the A.S.C.* In order to see if the production of A.S.C. was proportional to the size of dose following a single intraperitoneal injection, six pairs of 3-month-old male rats were injected with the dose range,

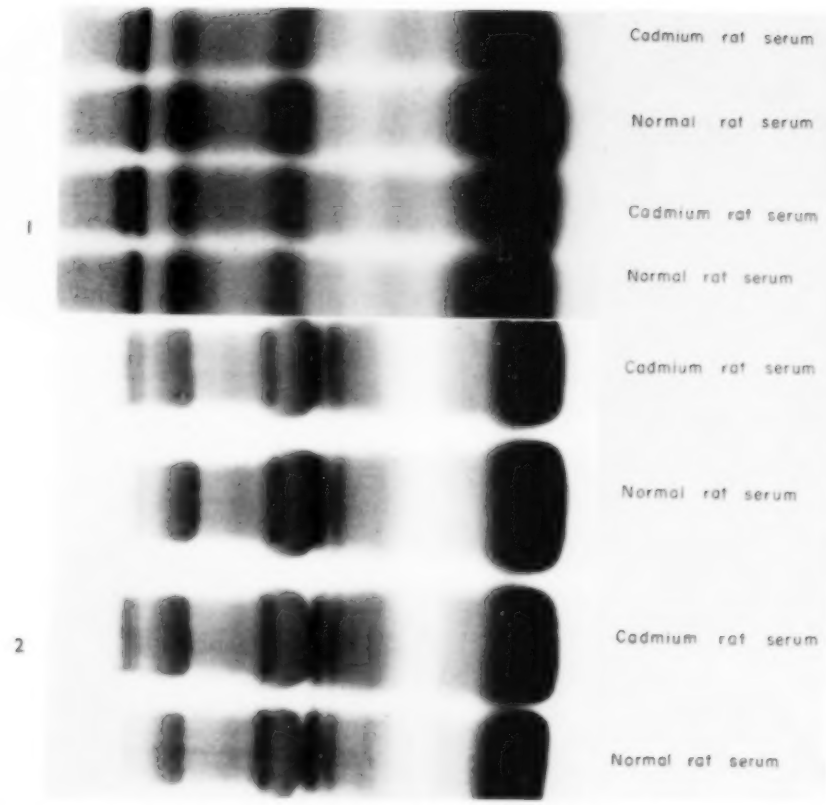


FIG. 2. Appearance of abnormal component in the sera of rats 24 hr after the intraperitoneal injection of 1 mg/kg of cadmium as cadmium chloride. (1) Continuous buffer system. (2) Discontinuous buffer system.

facing page 112

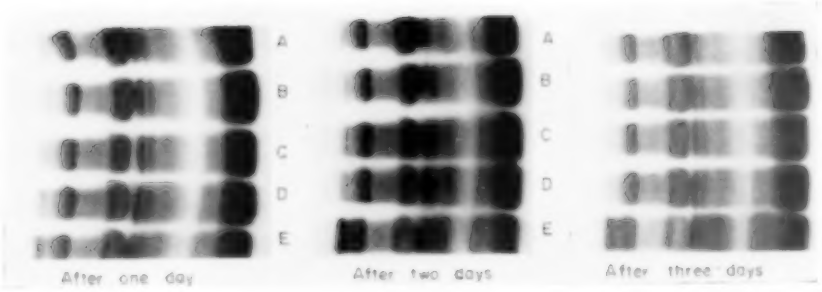


FIG. 3. Starch gel electrophoresis patterns of sera of rats given various daily doses of cadmium a cadmium chloride by intraperitoneal injection. A, normal rat serum. B, 0·025 mg cadmium/kg daily C, 0·100 mg cadmium daily. D, 0·200 mg cadmium daily. E, 1·000 mg cadmium daily.

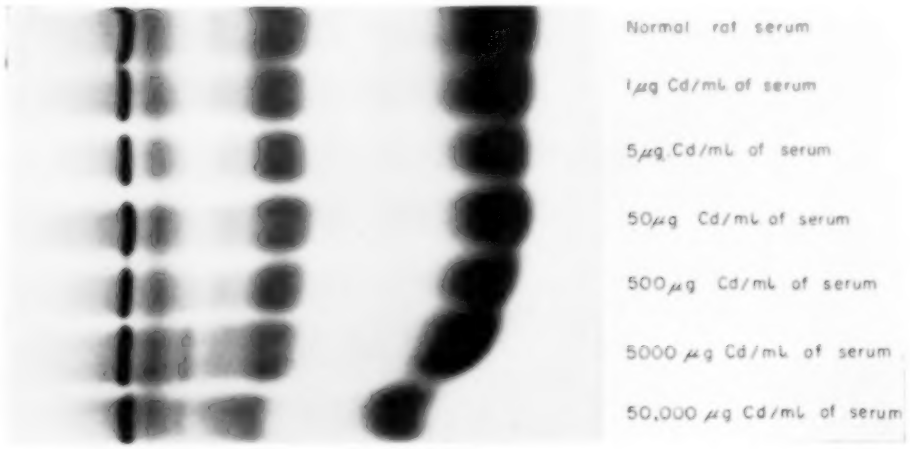

Normal rat serum
1 μg Cd/mL of serum
5 μg Cd/mL of serum
50 μg Cd/mL of serum
500 μg Cd/mL of serum
5000 μg Cd/mL of serum
50,000 μg Cd/mL of serum

FIG. 4. *In vitro* addition of cadmium to rat serum. Starch gel electrophoresis patterns of normal rat serum containing the indicated concentrations of cadmium (continuous buffer system).

0·5, 1·0, 1·5, 2·0, 2·5 and 3·0 mg/kg of inactive cadmium as cadmium chloride in saline. Blood was taken after 24 and 48 hr and the sera were subjected to starch gel electrophoresis, using the continuous buffer system.

After 24 and 48 hr, the animals given 2, 2·5 and 3 mg/kg of cadmium showed no A.S.C. line. The animals that received 0·5 and 1·5 mg/kg showed faint lines at 24 hr and slightly stronger ones at 48 hr. The animals that were given 1 mg/kg exhibited the strongest A.S.C. lines at 24 hr and even stronger ones at 48 hr. The optimum conditions for production of the A.S.C. line are therefore the administration of 1 mg/kg of cadmium and removal of the serum 2 days afterwards.

5. *Precipitation by ammonium sulphate*. Five samples of serum containing the A.S.C. were treated with saturated ammonium sulphate solution to give a range of saturation of 30–35–40–45–50 per cent. The precipitated sera were left at 0 °C for 1 hr and then centrifuged at 3000 rev/min for 20 min. The supernatant fluids were removed, dialysed free of the ammonium sulphate, and dialysed against normal saline prior to the electrophoresis. The precipitates were dissolved in 0·5 ml of distilled water and dialysed against water and finally saline. Starch gel electrophoresis using the continuous buffer system showed that at 35 per cent saturation of the serum, virtually all of the A.S.C. was precipitated, and at 40 per cent saturation, all of the A.S.C. was out of solution, within the limits of visibility of this technique.

In vitro addition of cadmium to normal serum

The delay of 24 hr following cadmium administration before the A.S.C. appeared in the serum, made it improbable that the A.S.C. was a result of the direct interaction between cadmium and an existing serum protein. This possibility was examined, however, by the following experiment. Inactive cadmium, as cadmium chloride, was added to normal rat serum so as to give the following concentrations: 1, 5, 50, 500, 5000 and 50,000 μg of cadmium per ml of serum. The mixtures were left for 1 hr in an incubator at 37 °C, then run on a starch gel, using the continuous buffer system. The only serum protein to be seriously affected was the albumin fraction, which migrated more slowly as the concentration of cadmium in the serum increased. The other bands in the serum appeared unaffected, and no line corresponding to the A.S.C. was observed (Fig. 4).

Formation of abnormal serum component by other metals

In order to determine whether the production of the A.S.C. was a specific symptom of cadmium absorption, four male rats were taken and given the following treatments:

Treatment 1. Saline daily for 3 days by intraperitoneal injection.
Treatment 2. Cadmium (1 mg/kg) daily for 3 days by intraperitoneal injection.
Treatment 3. Zinc (1 mg/kg) daily for 3 days by intraperitoneal injection.
Treatment 4. Mercury (1 mg/kg) daily for 3 days by intraperitoneal injection.

Blood was taken 1, 2 and 3 days from the commencement of dosing and the results revealed that the mercury administrations produced the A.S.C. in the blood rather more abundantly than cadmium, whereas zinc did not produce the band at the given dose rate. Much larger doses of zinc did, however, produce the A.S.C. A rat was given 20 mg/kg of zinc (as zinc acetate) daily for 2 days by intraperitoneal injection and 24 hr after the second dose, the animal was killed and samples of blood and peritoneal

fluid were collected. The injections had produced considerable fluid in the peritoneum and it was possible that the A.S.C. had originated from this site, with subsequent removal by the blood stream. Starch gel electrophoresis of the sera and peritoneal fluid using the continuous buffer system showed, however, that although the peritoneal fluid contained all the normal serum proteins in comparable amounts to the serum, its content of A.S.C. was very much smaller.

An extension of these experiments was made to observe the A.S.C.-producing capabilities of a range of metals. Pairs of adult male rats were given a single intraperitoneal injection of 1 mg/kg of the following metals: beryllium, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury and nickel. A further pair of rats served as controls and received a single injection of saline. The metals were administered as the acetate, and were neutralized before injection, with the exception of the beryllium salt, which was slightly acid. The animals were bled after 24 hr, the sera from each pair was pooled and examined by starch gel electrophoresis, using the continuous buffer system. The results showed (Fig. 5) that the intensity of the A.S.C. line produced varied considerably with the metal administered, in the following sequence:

mercury > cadmium, beryllium, copper > manganese

Faint bands were produced with chromium and nickel, barely perceptible bands with iron and cobalt and no visible band at all with lead.

Isolation of the abnormal serum component

The initial observation of the appearance of the A.S.C. posed many questions, and as a first step to answering some of them, a separation and partial purification of the material was attempted.

Apparatus. The electrophoretic separation described in this section was carried out using the apparatus described by Porath[13] and the procedure of Gedin and Porath.[14] The separation column consisted of a glass tube 150 cm in length and 2 cm internal diameter, surrounded by a 5 cm internal diameter cooling jacket. Purified cellulose (250 g) was well mixed with 2·2 l. of buffer and the column was carefully poured as directed. The homogeneity of the column was checked by pouring 2 ml of saturated DNP-aspartic acid, in buffer solution, in at the top and eluting down the column. The DNP-aspartic acid eluted out in 14 ml of solution, thereby indicating a satisfactorily packed column, which was then ready for use.

Buffers used. A borate–phosphate buffer, pH 8·4, and ionic strength 0·05, of the following composition was used in the experiments: 2·34 g of sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), 4·29 g of sodium tetraborate ($Na_2B_4O_7.10H_2O$), made up to 1 l. and degassed on a water pump 20 min before use.

Method. The column was used to effect an electrophoretic separation of the whole serum of rats injected with radioactive cadmium. Absorptiometer measurements and subsequent starch gel electrophoresis of the fractions showed that the abnormal serum component migrated at a slightly slower rate than the albumin fraction (Fig. 6). With this information, a sample of the material was isolated as follows.

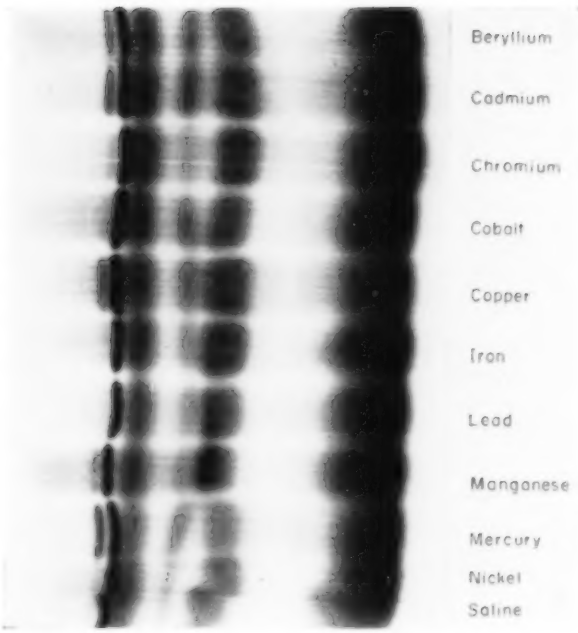


FIG. 5. Starch gel electrophoresis patterns of the sera of rats taken 24 hr after the intraperitoneal administration of 1 mg/kg of the metals indicated (continuous buffer system).

facing page 114

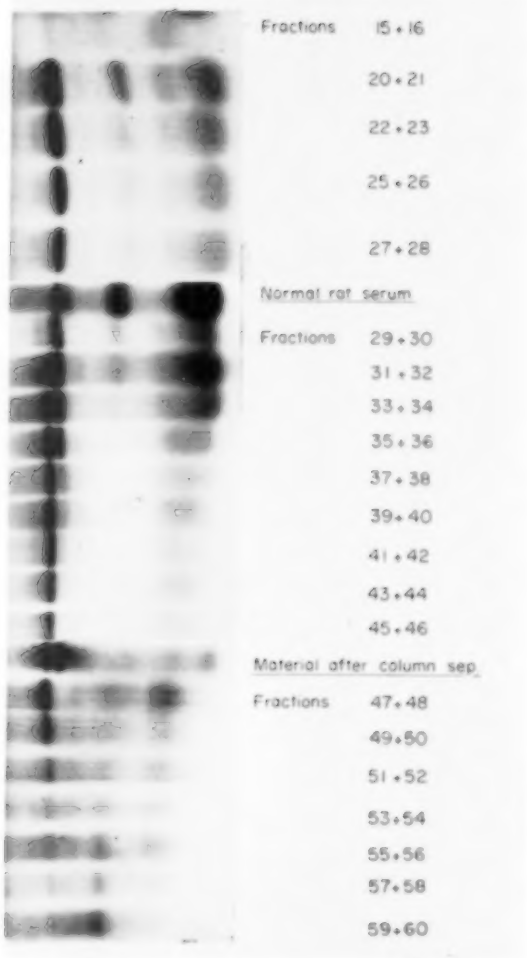


FIG. 7. Starch gel electrophoresis patterns of the freeze-dried fractions from the preparative zone electrophoresis column (continuous buffer system).

Thirty-six male adult rats, aged 3 months and weighing 320–350 g were given two doses of 1 mg/kg of radioactive cadmium on successive days, and were completely bled by cardiopuncture on the third day. The blood was left to clot for 1 hr at room temperature, put in the refrigerator at 0 °C for 2 hr and finally centrifuged at 3000 rev/min for 30 min. The serum was removed and twice its volume of distilled water was added, the resultant solution being left for 30 min, for haemolysis of the remaining red cells to take place. Saturated ammonium sulphate solution was then added to give a level of 40 per cent saturation. The mixture was left at 0 °C for 1 hr, and then spun at 3000 rev/min for 30 min. The precipitate was dissolved in saline, and dialysed against frequent changes of distilled water during the following day. Dialysis was continued until the water gave no reaction with barium chloride solution. The material was finally freeze-dried, and a sample reserved for future analysis. The freeze-dried material weighed 1·95 g.

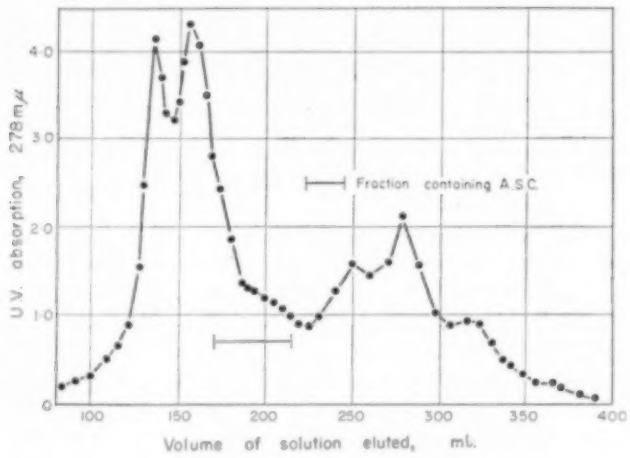


FIG. 6. Ultra-violet absorption curve. Fractions of cadmium rat serum from preparative zone electrophoretic column.

Some (1·9 g) of the freeze-dried material was dissolved in 22 ml of the borate–phosphate buffer, and centrifuged for 30 min before application to the column. The band of liquid 9·3 cm in depth, was drawn carefully into the cellulose medium, and the electrophoretic separation was allowed to run for 28 hr, at a voltage of 1200 V, and a current of 25 mA. At the end of the experiment, the column was eluted with the degassed borate–phosphate buffer solution and collected by a mechanical fraction cutter, in 4–5 ml fractions, at a rate of four per hr, until 400 ml had been eluted. This volume represented the hold-up volume of the column. The volumes of the samples were carefully measured and the u.v. absorption curve at 278 mμ was plotted. The samples were finally dialysed separately against distilled water for 3 days (three changes) at 0 °C, combined in pairs in the range fraction 15–86, freeze-dried and submitted to starch gel electrophoresis using the continuous buffer system. By examination of these gels (Fig. 7) it could be seen that the A.S.C. appeared at fraction 33 and

was almost absent after fraction 60. Fractions 35–52 (inclusive) were bulked together, dissolved in distilled water and freeze-dried. The final weight of the freeze-dried material was 620 mg and radioactivity measurements on the sample were negative.

The degree of purification affected by this method can be seen from Fig. 8, in which the material at stages after ammonium sulphate precipitation, and after the electrophoretic separation, were compared with a normal serum sample, by both continuous and discontinuous buffer systems. The two or three impurities observed in the starch gel analyses of the final material are, of course, serum proteins which migrate with the A.S.C. in the immediate post-albumin position in free electrophoresis.

Experiments with the isolated material

Electrophoresis on other media. Samples of the isolated A.S.C. were subjected to paper electrophoresis according to the method of Kunkel and Tiselius.[15] The material appeared to migrate at the same speed as in the column, that is, slightly slower than the albumin band, although serious trailing was observed. Attempts to use cellulose acetate strips in place of paper met with singularly little success, the material moving very slowly as an haphazard, very diffuse band, in both borate and barbitone buffers.

Sensitivity of starch gel electrophoresis. The sole criterion of the presence of A.S.C. in specimens of serum had, until this point, been its visibility as a stained band after starch gel electrophoresis. The following experiment was designed to determine the limit of sensitivity of this technique.

Five solutions of A.S.C. in saline were prepared, to give 1, 2, 3, 4 and 5 mg/ml of solution. These solutions, together with a normal serum, were subjected to starch gel electrophoresis using the continuous buffer system, and the stained gels were thoroughly washed for 3 days, with several changes of solution. The limit of visibility of the stained A.S.C. line was reached with the 2 mg/ml solution and therefore, the limit of sensitivity by this method was between 1 and 2 mg/ml of material.

Abnormal serum component—immunological studies

Preparation of antiserum to abnormal serum component. Although no A.S.C. has been observed in normal rat serum by the stained gel method, there was a distinct possibility that the substance was present in amounts too small to be detected. Obviously a more sensitive technique would be required before it could be definitely stated that this material was an abnormal component and not merely one which was already present in very small amounts.

Under suitable conditions the technique of immunological precipitation between antigen and antibody has been shown by many workers in other fields to be extremely sensitive. With this object in view, experiments were performed to determine whether the A.S.C. was antigenic, and if so, whether the antiserum so produced could be used as a sensitive method for the determination of small amounts of A.S.C. in serum.

Five male lop-eared rabbits, aged 7 months, were used for this experiment and were given the following injections:

Injection 1. 3 ml of oil (Bayol F), 1 ml of Arlacel A, 1 ml of sterile buffered saline, containing 12·5 mg of A.S.C., 12·5 mg of dried Canetti strain T.B. bacillus. The dried bacillus was ground up in the Bayol F oil, the remainder of the constituents added

Vol. 1961

ol. 7
961

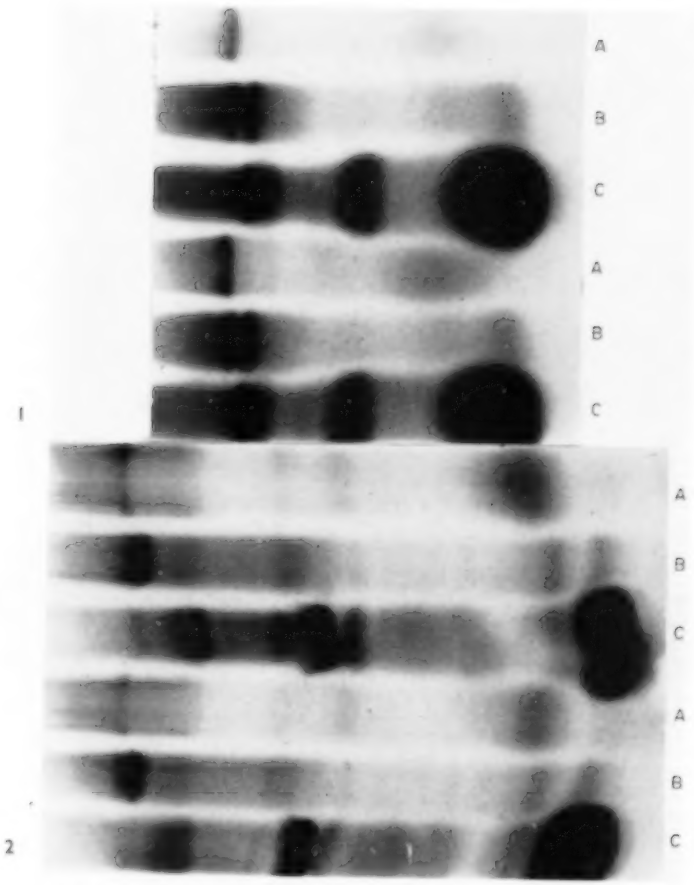


FIG. 8. Starch gel electrophoresis patterns of the material containing the abnormal serum component, at various stages of purification. (1) Continuous buffer system. (2) Discontinuous buffer system. A, final isolated material. B, cadmium serum after salt fractionation. C, normal rat serum.

facing page 116

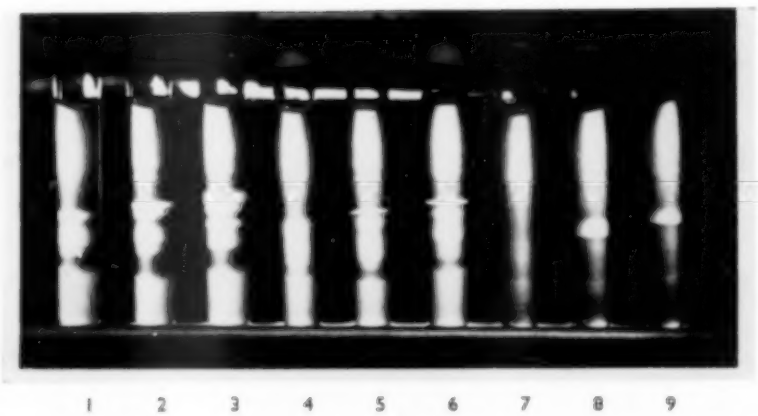


FIG. 9. Precipitin lines between antiserum and normal and cadmium rat serum. Top layer: 1, 4 and 7, normal rat serum; 2, 5 and 8, cadmium rat serum (*A*); 3, 6 and 9, cadmium rat serum (*B*). Bottom layer: 1, 2 and 3, 100 per cent rabbit antiserum (treated); 4, 5 and 6, 50 per cent rabbit antiserum (treated); 7, 8 and 9, 10 per cent rabbit antiserum (treated).

and the whole mixture thoroughly emulsified. A small volume (0·4 ml) of this solution was injected intramuscularly into each hind leg and the animals were left for 4 weeks.

Injection 2. 12·5 mg of A.S.C. in 0·3 ml of buffered sterile saline, 2·2 ml of aluminium phosphate gel. A small volume (0·4 ml) of this solution (equivalent to 2 mg of A.S.C.) was injected intravenously into each animal, two injections being given at an interval of 3 days. The animals were bled from the ear vein 1 and 2 weeks after the second injection and the serum obtained from these two bleedings was pooled and frozen in a deep freeze refrigerator until required. A volume of 170 ml of rabbit antiserum was obtained.

Precipitin reactions using the antiserum. The technique used in these experiments was that described by Oakley and Fulthorpe[16] with slight modifications. Ionagar no. 2 (Oxoid) and sterile buffered saline were used to prepare solutions containing 0·25% and 0·5% agar, incorporating 0·3% sodium azide as bacteriocide. Precipitin tubes of a suitable size were prepared with an inner film of agar and set up with three 1-cm layers of agar solution as follows: top layer, 0·1 ml of 0·5% agar solution, 0·1 ml of antigen solution; middle layer, 0·2 ml of 0·25% agar solution; bottom layer, 0·1 ml of 0·5% agar solution, 0·1 ml of antiserum. The layers were allowed to set, then stoppered, incubated at 37 °C and examined after 1, 3, 5, 7 and 14 days.

Precipitation of impurities. Starch gel electrophoresis of the isolated A.S.C. had already shown that two or three other serum components were visible. These proteins would, in all probability stimulate the production of antibodies when injected with the A.S.C. It was necessary, therefore, to precipitate the undesirable antibodies by the addition of suitable amounts of normal rat serum.

Varying amounts of normal rat serum were added to constant volumes of rabbit antiserum and the resultant solutions were tested for precipitin lines when matched against a solution of the A.S.C. in saline. It was found that the addition of 5% normal rat serum was sufficient to remove from the antiserum all the extraneous antibodies but one, whereas ten times this amount was necessary to afford complete removal of the remaining impurity. The addition of one part of normal rat serum to twenty parts of rabbit antiserum was considered sufficient for the purpose, allowance being made where necessary for the additional weak impurity.

Limit of detectibility of the A.S.C. An experiment to determine the optimum ratio of the A.S.C.–antibody system using the Oakley and Fulthorpe technique, showed that 1 ml of the rabbit antiserum reacted with 5 mg of A.S.C. at the optimum ratio. With this information, the limit of detectibility of the A.S.C., using the precipitin reaction, was estimated by the following procedure: Rabbit antiserum, to which 5 per cent of normal rat serum had been added, was matched with a 5 mg/ml solution of A.S.C. in order that optimum conditions should be obtained. Dilutions of A.S.C., using saline in one series and normal rat serum in the other, were matched against corresponding dilutions of the antiserum in saline (Table 1).

The limit of visibility in both series of tubes was the reaction occurring between 1 : 300 antiserum and the equivalent dilution of A.S.C. The limit of detectibility by this method then, is of the order of 16 μg/ml of A.S.C.

(The lines in tubes 16–20 were observed to be slightly lower than the corresponding

lines in tubes 6–10, and appeared to indicate the possibility that normal rat serum contained finite amounts of the A.S.C., thus causing depression of the band. This matter was examined in later experiments.)

Precipitin lines in cadmium rat serum. Two male rats *A* and *B* were given daily injections of 0·6 mg/kg of non-active cadmium by intraperitoneal injection for 3 weeks and at the end of this time the animals were bled by cardiopuncture.

Rabbit antiserum was treated with 5 per cent normal rat serum in the usual way and dilutions of this material giving 10 per cent, 50 per cent and 100 per cent of the original mixture were matched against serum obtained from a normal rat and also from rats *A* and *B* (Fig. 9).

The additional lines due to the presence of A.S.C. in the sera of rats *A* and *B* can readily be seen.

TABLE 1

Tube no.	Antiserum dilution (saline)	A.S.C. in saline (μg/ml)	Tube no.	Antiserum dilution (saline)	A.S.C. in rat serum (μg/ml)
1	1 : 1	5000	11	1 : 1	5000
2	1 : 20	250	12	1 : 20	250
3	1 : 40	125	13	1 : 40	125
4	1 : 60	83	14	1 : 60	83
5	1 : 80	62·5	15	1 : 80	62·5
6	1 : 100	50	16	1 : 100	50
7	1 : 160	31·3	17	1 : 160	31·3
8	1 : 200	25	18	1 : 200	25
9	1 : 300	16·7	19	1 : 300	16·7
10	1 : 500	10	20	1 : 500	10

The possibility that the A.S.C. had generated an auto-immune antibody to itself was examined by matching sera from rats *A* and *B* and a normal rat, against solutions containing 5, 1 and 0·1 mg/ml of A.S.C. in saline. A diffuse band was observed in all the tubes after 3 days, but the reactions between *A* and *B* and the A.S.C. were seen to be much stronger than those observed between the normal rat serum and the A.S.C. After 7 days, all the bands had disappeared.

The experiment was extended to examine the reactions of normal rat serum and combined sera from *A* and *B*, with dilutions of A.S.C. in saline to give 50, 10, 5, 1 and 0·1 μg/ml of A.S.C.

Two weaker bands were visible in those tubes containing normal rat serum, but two much stronger bands in a similar position were observed in the tubes containing serum from rats treated with cadmium. Gradual disappearance of the bands with time was observed as in the previous experiment. No explanation of this phenomenon is offered.

Immunoelectrophoresis

The isolated A.S.C. fraction contained several trace amounts of normal rat serum protein and by means of the technique of immunoelectrophoresis, the position of the various antigen–antibody reactions resulting from these impurities could be seen in relation to the main A.S.C.–A.S.C. antibody precipitin line.

Immunoelectrophoresis between A.S.C. and the rabbit antiserum. A solution of 10 mg/ml of the isolated A.S.C. in saline was subjected to starch gel electrophoresis using the continuous buffer system. The resultant gel was sectioned, one part being stained with naphthalene black solution, and the other being laid face downwards on a suitably prepared agar gel. Strips of filter paper were dipped in the rabbit antiserum, blotted damp dry and applied to the agar gel as previously described. After 7 days the following pattern was observed (Fig. 10). Accurate measurements of the gel showed that the A.S.C.–A.S.C. antibody reaction occurred at point *A*, other precipitin lines being due, presumably, to reactions between impurities in the A.S.C. material, and corresponding antibodies in the rabbit antiserum.

Precipitin reactions using the A.S.C. antiserum. Since Öuchterlony[17] introduced a method for the observation of precipitin lines by their formation in an agar gel contained in a glass Petri dish, this principle has found wide application in bacteriology and immunochemistry. A micro-modification of this method by Öuchterlony and Wadsworth,[18] using agar-coated glass microscope slides, has the advantage of conserving material and giving a result in a shorter time.

Effect of adding normal rat serum to rabbit antiserum. The Öuchterlony plate technique was used to examine the effect of addition of increasing volumes of normal

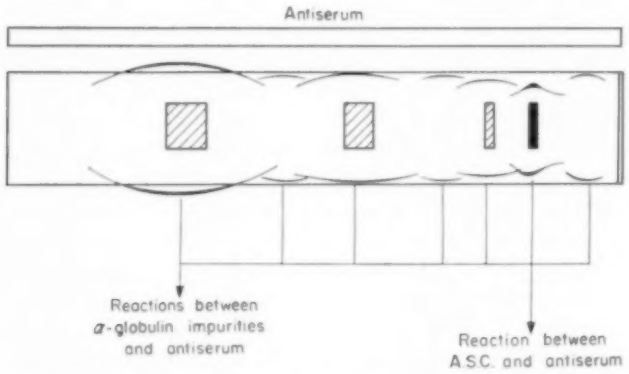


FIG. 10. Immunoelectrophoresis: precipitin bands between the isolated abnormal serum component and its rabbit antiserum.

rat serum to the rabbit antiserum, when the latter was matched against a solution of A.S.C. A solution of 3 mg/ml of A.S.C. was placed in the centre hole, and in the surrounding six holes, appropriate volumes of the following mixtures (Fig. 11): (1) Rabbit antiserum. (2) Rabbit antiserum (1 vol.) plus normal rat serum (0·05 vol.). (3) Rabbit antiserum (1 vol.) plus normal rat serum (0·5 vol.). (4) Rabbit antiserum (1 vol.) plus normal rat serum (1 vol.). (5) Rabbit antiserum (1 vol.) plus normal rat serum (3 vols.). (6) Normal rat serum.

It can be seen that there are multiple lines between the A.S.C. in the centre and the untreated antiserum. The addition of 0·05 vol. of normal rat serum to 1 vol. of rabbit antiserum left only two lines, and the addition of 0·5 vol. of normal rat serum to 1 vol. of rabbit antiserum gave a single line which did not appear to diminish in

intensity with increasing concentrations of normal rat serum. No line was formed between the rat normal serum and the A.S.C. solution.

Precipitin lines following varying intradermal doses of cadmium. Three rats were given single injections of 1, 2 and 3 mg/kg of inactive cadmium, as cadmium chloride, intradermally, blood specimens being taken after 24 hr. The sera from these rats, together with normal rat serum and a 0·5 mg/ml. solution of A.S.C. were matched on a micro-Öuchterlony plate against a 1 : 30 dilution of rabbit antiserum to which 5 per cent normal rat serum had been added. All the specimens gave precipitin lines except the normal rat serum.

Precipitin lines following low intraperitoneal doses of cadmium. Three rats were given single injections of 10, 50 and 100 μg/kg of inactive cadmium, as cadmium chloride, by intraperitoneal injection, saline being administered to a control rat. On examination of the sera after 24 hr, using the micro-Öuchterlony technique, easily visible lines were observed between the antiserum and the sera of the rats receiving 50 and 100 μg/kg. An almost imperceptible line was visible at the junction between the antiserum and the serum of the rat given a dose of 10 μg/kg. Evidence of the production of the A.S.C. can therefore be demonstrated after the injection of between 10 and 50 μg/kg of cadmium.

Vol. 7
1961

DISCUSSION

The introduction of the technique of electrophoresis of proteins has stimulated a great deal of research in experimental and clinical medicine, notably in attempts to correlate possible deviations from the normal body proteins with the progress of the particular disease or disorder under study. In addition, the modern techniques of ultracentrifugation and immunochemistry have been applied to this problem.

The investigation of possible serum protein changes in the rat following the administration of soluble cadmium salts, was initially conducted by the author using the method of filter paper electrophoresis. It soon became obvious, however, that although gross changes in the serum protein pattern could be identified in this way, a more sensitive method would be required to observe smaller deviations from the normal. It was decided to make use of the horizontal starch gel technique devised by Smithies,[12] and this method was used almost exclusively in the investigations reported in this paper.

An abnormal serum component was observed 24 hr after the intraperitoneal administration of a single dose of cadmium. Previous experiments[19] had shown that the level of cadmium in the blood following a single intraperitoneal injection, dropped to a negligible value after 5 hr, but, nevertheless, the possibility that the abnormal component was the result of a direct cadmium–serum combination was examined by the addition of cadmium to normal rat serum *in vitro*. The results indicated a progressive lowering of the migration rate of the albumin fraction, from which cadmium–albumin binding may be inferred, but no band comparable with the abnormal serum component was seen on starch gel electrophoresis of the sera from this experiment. At the time of production of the abnormal serum component, that is, 20–24 hr after the injection, the administered cadmium had already been laid down in the tissues of the body, and 50 per cent of this cadmium could be found in the liver. It seems possible

that the abnormal serum component is the result of aberrated biosynthesis of the serum proteins, resulting from the action of cadmium on one or more of the liver constituents.

Further evidence for this theory is afforded by the difficulty experienced in separating a sample of the abnormal serum component from the serum protein migrating immediately in front of the abnormal component in starch gel electrophoresis, using the continuous buffer system. Earlier experiments using British Drug Houses Ltd. Soluble Starch, appeared to indicate a complimentary role for these two components, i.e. as the abnormal component increased, the adjacent protein decreased. This phenomenon was not so apparent in the present experiments, but would probably repay further investigation.

Another interesting aspect of this work was the observation that at relatively high doses of, for example, 2 mg/kg of cadmium, the abnormal serum component was not produced. If the action of low doses of cadmium results in an abnormal biosynthesis, then higher levels of cadmium may well prove completely cytotoxic to the cells concerned in the production of the abnormal component.

In addition to cadmium, several other metals, when injected intraperitoneally, caused the production of the abnormal serum component. Mercury, indeed, appeared more effective, than cadmium, whereas beryllium and copper were equally effective, manganese, chromium and nickel somewhat less effective, lead giving no comparable reaction at all. Stokinger and Wagner[10] have reported that the α-globulin fractions of the sera of dogs and rabbits were elevated following exposure to small doses of cobalt. This finding may possibly be related to the faint band found in the rats receiving cobalt, although the serum neuraminic acid levels were normal, as opposed to the elevated levels found by Stokinger and Wagner. This point obviously requires further investigation.

Electrophoretic analysis has shown that a large range of different diseases, having a common syndrome of malnutrition and wasting, tend to give a similar overall serum pattern.[20] Such diseases as nephrosis, cirrhosis, hepatitis, multiple myeloma, syphilis, tuberculosis, sarcoid, leprosy, malaria, lobar pneumonia, rheumatic fever, rheumatoid arthritis and lupus erythrematosis disseminatus, have all been shown to produce a lowered albumin content and an increased globulin content of the plasma. General observations of altered plasma protein distribution with the occurrence of various diseases, are of somewhat limited value, unless extended to include a more detailed study of specific changes. The production of an abnormal serum component by several metals, and cadmium in particular, can be considered rather different from the serum changes apparent in these diseases, especially in view of the very small doses required to produce it, and the comparatively short time before its appearance in the serum.

Acknowledgements—The author is indebted to Dr. Donald Hunter and Dr. A. I. G. McLaughlin for interest and encouragement with the work. Thanks are also gratefully extended to Professor C. J. O. R. Morris and Dr. R. G. White for advice and assistance with the techniques of electrophoresis and immunology, respectively, and to Miss Kathleen Ballard and Mr. B. J. Biles for valuable technical help.

REFERENCES

1. E. W. Schwartze and C. L. Alsberg, *J. Pharmacol.* **21**, 1 (1923).
2. C. O. Johns, A. J. Finks and C. L. Alsberg, *J. Pharmacol.* **21**, 59 (1923).
3. O. G. Fitzhugh and F. H. Meiller, *J. Pharmacol.* **72**, 15 (1941).
4. R. H. Wilson and F. DeEds, *Arch. Industr. Hyg.* **1**, 73 (1950).

5. L. E. DECKER, R. U. BYERRUM, C. F. DECKER, C. A. HOPPERT and R. F. LANGHAM, *Arch. Industr. Hlth.* **18**, 228 (1958).
6. D. J. LAWFORD, *Nature, Lond.* In press.
7. R. TRUHAUT and C. BOUDENE, *Arh. Hig. Rada* **5**, 19 (1954).
8. M. BARNI, M. DE FELICE and L. REALE, *Folia Med.* **41**, 168 (1958).
9. C. P. ODESCALCHI, P. ANDREUZZI and A. T. VILLA, *Folia Med.* **41**, 1179 (1958).
10. N. E. STOKINGER and W. C. WAGNER, *Arch. Industr. Hlth.* **17**, 273 (1958).
11. M. D. POULIK, *Nature, Lond.* **180**, 1477 (1957).
12. O. SMITHIES, *Biochem. J.* **61**, 629 (1955).
13. J. PORATH, *Biochim. Biophys. Acta* **22**, 151 (1956).
14. H. I. GEDIN and J. PORATH, *Biochim. Biophys. Acta* **26**, 157 (1957).
15. G. H. KUNKEL and A. TISELIUS, *J. Gen. Physiol.* **35**, 89 (1951).
16. C. L. OAKLEY and A. J. FULTHORPE, *J. Path. Bact.* **65**, 49 (1953).
17. Ö. OUCHTERLONY, *Acta Path. Microbiol. Scand.* **32**, 231 (1953).
18. Ö. OUCHTERLONY and C. WADSWORTH. Unpublished, referred to in *Progress in Allergy* Vol. 5. Karger, Basle, Switzerland.
19. D. J. LAWFORD, *J. Lab. Clin. Med.* In preparation.
20. A. B. GUTMAN, *Advanc. Protein Chem.* **4**, 155 (1948).

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7 pp. 123-134. Pergamon Press Ltd., Printed in Great Britain.

INHIBITION BY ANTITUMOUR AGENTS OF LABELING OF NUCLEAR PROTEINS *IN VIVO* WITH L-ARGININE-U-^{14}C

HARRIS BUSCH, DOLORES C. FIRSZT, ALLAN LIPSEY, ELLI KOHEN and S. AMER

Departments of Biochemistry and Pharmacology, Baylor University College of Medicine, Houston, Texas

(*Received* 3 *December* 1960)

Abstract—Arginine-U-^{14}C was injected into animals bearing the Walker tumor at various times after treatment with one of a series of antitumor agents, including Myleran®, Leukeran®, 5-fluorouracil, 6-mercaptopurine, podophyllotoxin, colchicine and HN2. The tissues were excised 1 hr later and the labeling of proteins in various cellular fractions was determined. In the Walker tumor, the inhibition of labeling of the acid-insoluble nuclear proteins was the only constant effect of these agents. Podophyllotoxin suppressed the labeling of the nuclear proteins to a greater extent than the labeling of proteins of other fractions of the Ehrlich ascites tumor; however, colchicine did not exert a selective effect on labeling of the nuclear proteins. The effects on labeling of the proteins of the liver and spleen varied from inhibitor to inhibitor. From these data it would appear that the inhibition of labeling of the acid-insoluble nuclear proteins is an intermediate stage of the inhibitory activity of these compounds.

EXPERIMENTS recently reported from this laboratory[1] have indicated that the mustard, 5-bis(2-chloroethyl)aminouracil exerted a marked suppressive effect on the incorporation of L-arginine-U-^{14}C into the nuclear proteins of the Walker 256 carcinosarcoma, particularly the acid-insoluble nuclear proteins. Remarkable inhibitory effects were also noted with respect to the labeling of the acid-insoluble nuclear proteins of the spleen. In both tumor and spleen the labeling of the histones was also suppressed, but the labeling of the cytoplasmic proteins was not affected. In recent studies with a number of mustards of amino acids, Nyhan and Pula[2] noted that the labeling of the proteins of a variety of cellular fractions was markedly suppressed and the specific effects found with the aminouracil mustard did not seem to be a general phenomenon for the mustards. The present experiments were designed to extend the studies previously reported to a variety of types of antitumor agents, including mustards, sulfonoxy compounds, and purine and pyrimidine analogues.

The doses of the agents used in these studies were those reported to exert suppressive effects on the growth of tumors *in vivo*. At intervals of 23 and 47 hr after treatment with the drug, 10 μc of L-arginine-U-^{14}C were injected into the tumor-bearing animals. The tissues were treated as previously indicated,[3, 4] to ascertain the extent of inhibition of labeling of the proteins of various intracellular fractions. The drugs employed included 6-mercaptopurine, 5-fluorouracil, Myleran,® Leukeran® (Chlorambucil), HN2, colchicine and podophyllotoxin. The results obtained provide evidence that

* These studies were supported in part by grants from the National Cancer Institute, the Jane Coffin Childs Fund and the American Cancer Society.

the suppression of incorporation of L-arginine-U-^{14}C into nuclear proteins is a common effect of a variety of effective antitumor agents. However, this effect is usually accompanied by simultaneous suppression of incorporation of this amino acid into other cellular fractions.

MATERIALS AND METHODS

For the most part, the procedures employed in the present study were those used previously.[1, 3, 4] The agents employed in this study and their doses were as follows: 5-fluorouracil, 25 mg/kg;[5] 6-mercaptopurine, 50 mg/kg and 100 mg/kg;[5] Leukeran® (chlorambucil) 15 mg/kg and 30 mg/kg;[7–9] Myleran®, 15 mg/kg and 30 mg/kg;[10] HN2, 2 mg/kg;[11] colchicine[12] and podophyllotoxin.[13] These agents were intraperitoneally administered, in dimethyl-formamide,[11] at intervals of 23 and 47 hr, respectively, prior to the administration of the labeled arginine. One hour after the injection of the arginine-U-^{14}C, the animals were exsanguinated and the tissues were treated as previously described.[3,4] Nuclear preparations of Ehrlich ascites cells were made by the method recently described.[4] However, twenty-five strokes of the pestle through the fluid were necessary for disrupting these cells. In those instances in which the results with higher doses were not significantly different from the results obtained with the lower doses, the data in the figures are for the lower doses. The tumors were implanted from 6 to 8 days prior to injection of the drug. The tumors ranged in diameter from 0·5–1·0 cm when Walker tumors were used. The total ascitic fluid ranged from 3 to 6 ml when ascites tumors were used.

Vol. 7
1961

RESULTS

Leukeran

The effects of Leukeran® on the uptake of amino acids into the various cellular proteins of the Walker 256 carcinosarcoma and the spleen are indicated in Figs. 1 and 2. As was found with the aminouracil mustard,[1] the specific activities of the proteins of the liver were not influenced by the drug. In doses of either 15 or 30 mk/kg, Leukeran® exerted a marked suppressive effect on the labeling of both nuclear and cytoplasmic fractions of the Walker tumor (Fig. 1). The specific activities of the nuclear proteins were approximately 30 per cent of control values at 24 hr after the administration of Leukeran.® The specific activities of the proteins were 34 per cent, 44 per cent and 44 per cent of control values in the microsomal fraction, mitochondrial fraction and the cytoplasmic sap, respectively. This drug did not produce the selective suppression of labeling of the nuclear proteins in the tumor which was found with the aminouracil mustard. On the other hand, the results for spleen (Fig. 2) were much more like those found with the Walker tumor in the previous studies with the aminouracil mustard.[1] The specific activities of the nuclear proteins were markedly suppressed at 24 hr after the administration of the drug, i.e. to approximately 21 per cent of control values. On the other hand, the specific activity of the proteins of the cytoplasmic fractions were reduced to 60 per cent, 75 per cent and 81 per cent, respectively, of control values for proteins of the cytoplasmic sap, mitochondrial proteins, and microsomal proteins.

HN2

Nyhan and Pula[2] and Nyhan[14] have reported the similarity of some aspects of their results with HN2 with the results reported from this laboratory on the effects of the

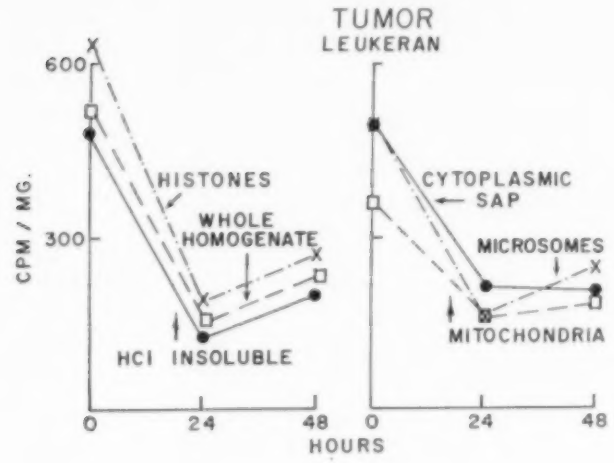


FIG. 1. Effect of Leukeran® on labeling of the proteins of the Walker tumor. In these experiments, 15 mg/kg of Leukeran® was administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment, 10 μc of L-arginine-U-^{14}C were injected, into the tumor-bearing rat. At the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

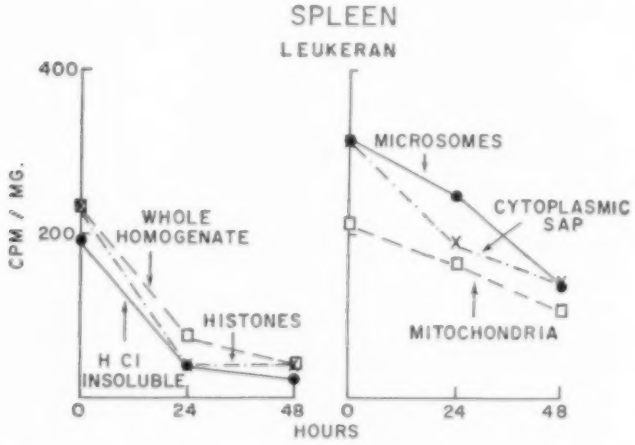


FIG. 2. Effect of Leukeran® on labeling of the proteins of the spleen. In these experiments, 15 mg of Leukeran® per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment, 10 μc of L-arginine-U-^{14}C were injected into the tumor-bearing rat. At the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

aminouracil mustard on the uptake of labeled arginine into proteins of the Walker tumor. Similar results have obtained in the present experiments (Table 1) with the Walker tumor. Similarly, in the spleen, the decrease in the specific activity of the proteins of the cytoplasmic fractions was not marked at 24 hr after administration of the drug. However, the labeling of the histones was suppressed to 50 per cent of control values. These results are similar to those obtained with other mustards. As was found previously,[1] an increase was noted in the specific activity of proteins of the liver following treatment of the animal with nitrogen mustard.

Myleran

The third of the alkylating agents included in this study was Myleran®, a compound which did not markedly suppress the growth of the tumor in the time period studied.

TABLE 1. SPECIFIC ACTIVITIES OF CELLULAR PROTEINS AFTER TREATMENT OF THE ANIMAL WITH HN2

At 23 hr following injection of 2 mg of HN2 per kg, arginine-U-^{14}C was injected intraperitoneally and the tissues were treated as described in the text. The values presented are averages of two experiments. The data for individual experiments are in the parentheses. The values are counts/min per mg dry weight of protein.

Protein fractions	Tumor		Liver		Spleen	
	counts/min per mg	% of control	counts/min per mg	% of control	counts/min per mg	% of control
Whole homogenate	277 (242,312)	67	471 (452,489)	183	187 (164,209)	75
Mitochondria	268 (258,279)	74	542 (490,594)	204	366 (360,372)	166
Microsomes	398 (395,400)	81	739 (700,778)	170	342 (266,417)	100
Cytoplasmic sap	365 (350,380)	74	421 (360,482)	199	316 (302,329)	100
Histones	354 (320,387)	56	364 (348,380)	200	135 (130,139)	53
HCl insoluble nuclear proteins	322 (295,348)	67	360 (359,360)	188	179 (173,185)	95

The specific activities of the nuclear proteins of the Walker tumor (Fig. 3), spleen (Fig. 4), and liver (Fig. 5) were decreased to different extents following the administration of the drug. The specific activities of the HCl-insoluble nuclear proteins and the histones of the tumor (Fig. 3) were reduced to 41 per cent and 54 per cent of control values, respectively at 24 hr after administration of a dose of 15 mg/kg of Myleran®. The decreases in the specific activities of the cytoplasmic fractions were markedly less at 24 hr. These results for the tumor were consistent with those obtained previously with the aminouracil mustard.[1] However, the very marked effect of the mustards, i.e. HN2, the aminouracil mustard and Leukeran®, on the labeling of nuclear proteins of the spleen (Fig. 4) was not noted with Myleran®. In the first 24 hr

Vol. 7
1961

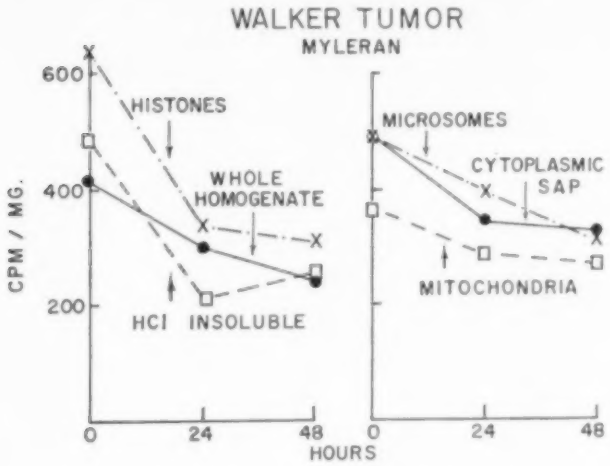


FIG. 3 Effect of Myleran[10] on labeling of the proteins of the Walker tumor. In these experiments, 15 mg of Myleran[10] per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment, 10 μc of L-arginine-U-^{14}C were injected into the tumor-bearing rat. At the termination of the experiment the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

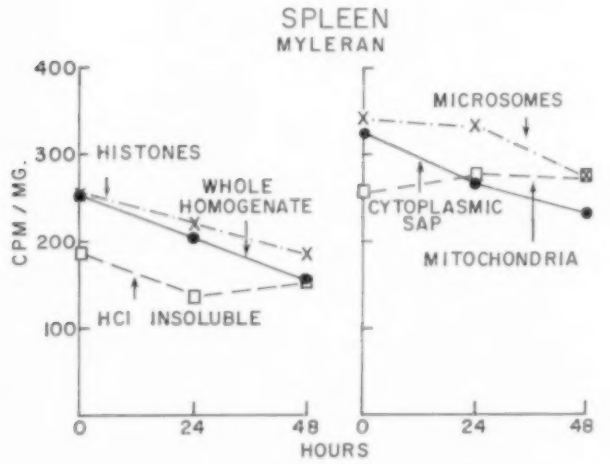


FIG. 4. Effect of Myleran[10] on labeling of the proteins of the spleen. In these experiments, 15 mg of Myleran[10] per kg were administered intrapertitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment, 10 μc of L-arginine-U-^{14}C were injected into the tumor-bearing rat. At the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

after administration of Myleran®, there was a marked effect on the labeling of the proteins of the liver which was not found with the mustards. The specific activity of the HCl-insoluble nuclear proteins decreased to 45 per cent of control values and similar suppressive effects were noted on labeling of the proteins of the microsomal fraction. By 48 hr, some recovery of the incorporating reactions was evident in the tumor and liver.

5-Fluorouracil

Inasmuch as each of the alkylating agents exhibited some specificity of effect in suppression of the incorporation of arginine-U-^{14}C into the nuclear proteins of the Walker tumor, the corresponding studies were made on analog inhibitors, i.e. 5-fluorouracil and 6-mercaptopurine. In the Walker tumor (Fig. 6), 5-fluorouracil suppressed labeling of histones to 60 per cent of control values and suppressed labeling of acid-insoluble nuclear proteins to 70 per cent of control values at 24 hr after administration of the drug. At 48 hr after administration of the drug, the labeling of the histones was suppressed to 50 per cent of control values and the labeling of the HCl-insoluble nuclear proteins was suppressed to 32 per cent of control values. In contrast, the labeling of the mitochondria was virtually unaffected.

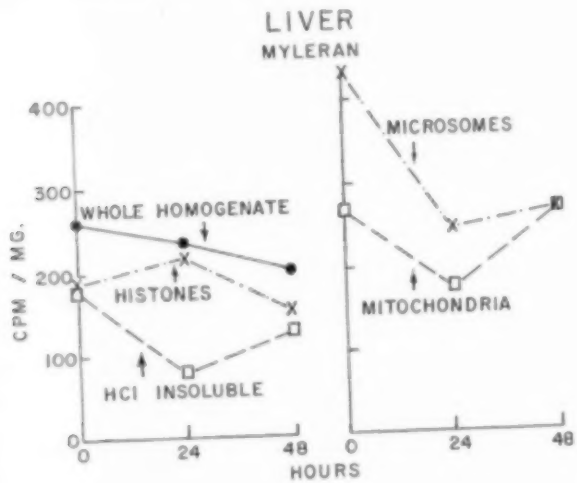


FIG. 5. Effect of Myleran[10] on labeling of the proteins of the liver. In these experiments, 15 mg of Myleran[10] per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment, 10 μc of L-arginine-U-^{14}C were injected into the tumor-bearing rat. At the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

The specific activities of the proteins of the microsomes and cytoplasmic sap were reduced to approximately 60 per cent of control values at 48 hr after administration of the drug. 5-Fluorouracil also suppressed the labeling of the nuclear proteins of the spleen and the liver of the tumor-bearing animal. The specific activity of the histones of the spleen (Fig. 7) was reduced to 40 per cent of control values by 24 hr following

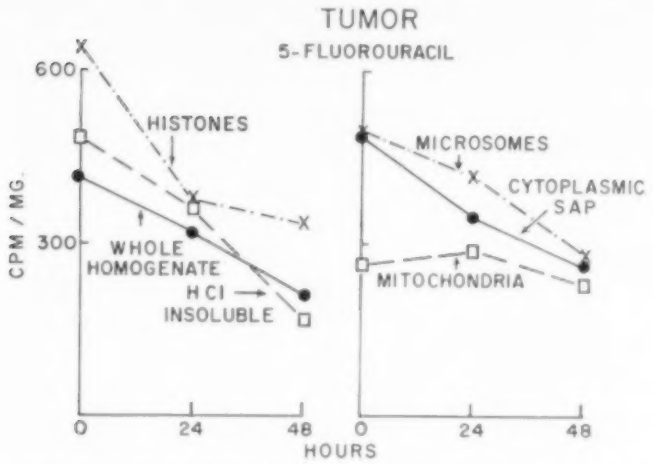


FIG. 6. Effect of 5-fluorouracil on labeling of the proteins of the Walker tumor. In these experiments, 25 mg of 5-fluorouracil per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

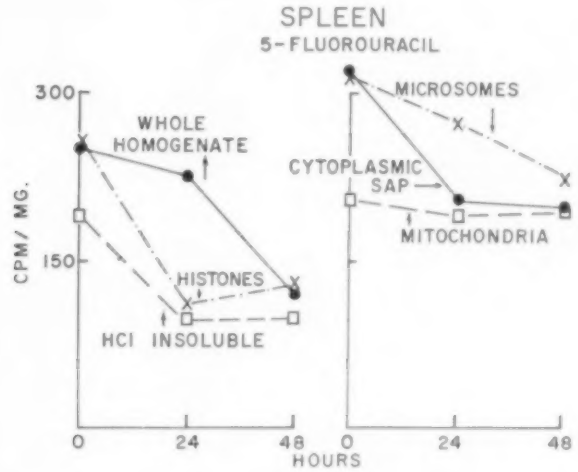


FIG. 7. Effect of 5-flourouracil on labeling of the proteins of the spleen. In these experiments, 25 mg of 5-fluorouracil per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

the administration of the fluorouracil and the specific activity of the acid-insoluble nuclear proteins was reduced to 50 per cent of control values at that time. The specific activities of the cytoplasmic proteins were also decreased but to a lesser extent. In the liver (Fig. 8), the specific activities of the proteins of each of the fractions were

suppressed following administration of the drug. The inhibitory effect was almost uniform in the fractions of the liver as contrasted to the other tissues studied.

6-*Mercaptopurine*

At 24 hr following the administration of 6-mercaptopurine, the most marked effect in the tumor (Fig. 9) was the suppression of labeling of the acid-insoluble

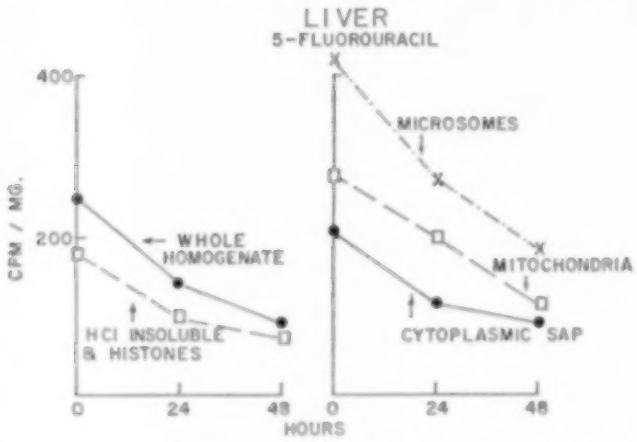


FIG. 8. Effect of 5-fluorouracil on labeling of the proteins of the liver. In these experiments, 25 mg of 5-fluorouracil per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

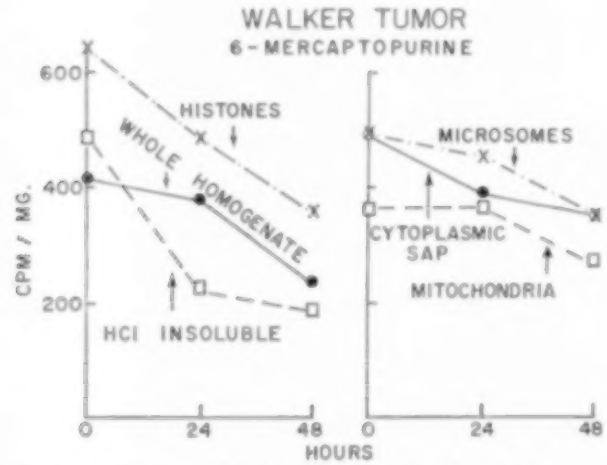


FIG. 9. Effect of 6-mercaptopurine on labeling of the proteins of the Walker tumor. In these experiments, 50 mg of 6-mercaptopurine per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. At the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three to four experiments.

Vol. 7
1961

nuclear proteins. The labeling of these proteins was suppressed to approximately 40 per cent of control values. Suppression of labeling of the cytoplasmic fractions was not marked at either 24 or 48 hr following the administration of this agent. The suppression of labeling of the histones continued during the second 24 hr; thus, the specific activity of the histones at 48 hr after administration of the drug was 55 per cent of control values. Significant changes were not found in the specific activities of the proteins of various fractions of the spleen (Fig. 10) at 24 hr after administration of the drug. In the second 24 hr, there was a suppression of labeling of each of the fractions of the spleen to 60–70 per cent of control values. In the liver (Fig. 11), the specific activity of the nuclear proteins was decreased to 70 per cent of control values at 24 hr after administration of 6-mercaptopurine. The uptake of the isotope into the proteins of the mitochondria and the cytoplasmic sap was also about 70–80 per cent of the control values at 24 hr after administration of the drug. The labeling of the microsomal proteins was decreased to about 60 per cent of control values at that time. In the second 24 hr, there was no marked change in the specific activity of these proteins.

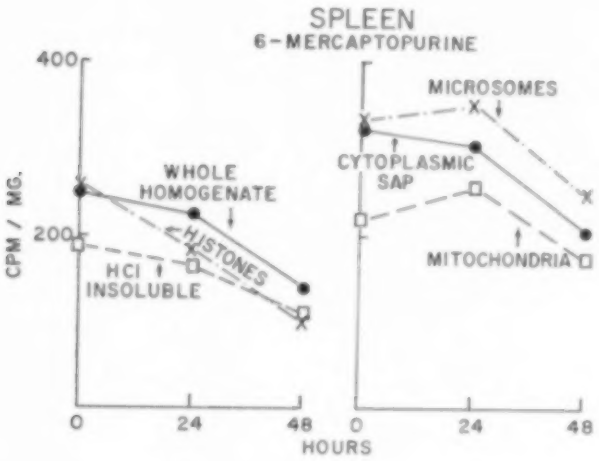


FIG. 10. Effect of 6-mercaptopurine on labeling of the proteins of the spleen. In these experiments, 50 mg of 6-mercaptopurine per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. At the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three to four experiments.

Colchicine

The data presented in Table 2 indicate that colchicine suppresses the uptake of isotope of L-arginine-U-^{14}C into proteins of the Ehrlich ascites tumor. The suppression of labeling of the nuclear proteins was equivalent to that of the proteins of the whole homogenate. With this compound, the relative suppression of labeling of the cytoplasmic proteins exceeded that of the nuclear proteins.

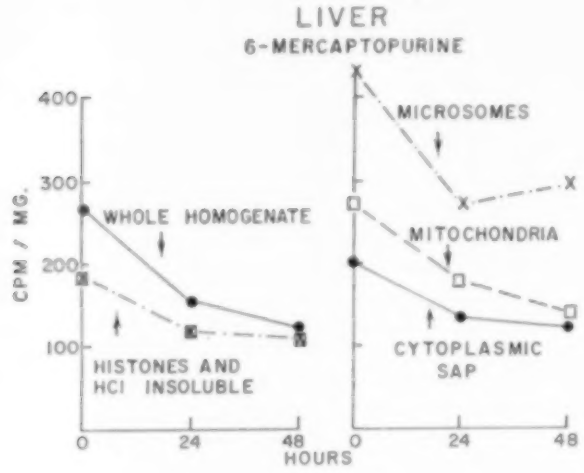


FIG. 11. Effect of 6-mercaptopurine on labeling of the proteins of the liver. In these experiments, 50 mg of 6-mercaptopurine per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. At the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three to four experiments.

TABLE 2. EFFECTS OF COLCHICINE AND PODOPHYLLOTOXIN ON LABELING OF NUCLEAR PROTEINS OF EHRLICH ASCITES CELLS

Fractions	Controls[4] Average ± standard error	Colchicine[12] Average ± standard error	Podophyllotoxin[3] Average ± standard error
Whole homogenate	4160 ± 590	2630 ± 480	2370 ± 500
Acid insoluble	4260 ± 500	2895 ± 370	2660 ± 580
Histones	5700 ± 680	3850 ± 760	2630 ± 595
Microsomes	3090 ± 1000	1320 ± 320	1690 ± 300
Mitochondria	1860 ± 280	920 ± 140	1320 ± 360
Cytoplasmic	3230 ± 1340	1130 ± 310	1770 ± 440

In the experiments with colchicine, 2 μg of the drug were injected into the ascites fluid *in vivo* 24 hr prior to the injection of 2·5 μc of the L-arginine-U-^{14}C. After 1 hr, the cells were removed and treated as previously described[3, 4] for other tissues. In experiments with podophyllotoxin glucoside, 2 mg of the drug were injected into the Ehrlich ascites fluid *in vivo*, 18 hr prior to the experiment. The values for control experiments, experiments with colchicine and experiments with podophyllotoxin are averages of five, eight and four individual determinations, respectively. The *P*-value for depression of the uptake of isotope into mitochondria in the experiments with colchicine was less than 0·05. The *P*-value for depression of uptake of isotope into histones in the experiments with podophyllotoxin was also less than 0·05. The standard error was determined from the equation: $E = \sqrt{\{\Sigma d^2/[n(n-1)]\}}$.

Podophyllotoxin

Marked suppression of the labeling of proteins of the Ehrlich ascites tumor was also found with podophyllotoxin. With the conditions employed, the specific activities of the nuclear proteins were suppressed to 47 per cent of control values. The suppression of labeling of the proteins of the cytoplasmic fractions was not as marked as the suppression of labeling of the nuclear proteins.

DISCUSSION

Perhaps the most interesting feature of the present study is the consistent suppression of labeling of the acid-insoluble nuclear proteins by the effective antitumor agents studied. In addition to the animouracil mustard, Myleran®, Leukeran®, HN2, 5-fluorouracil, and 6-mercaptopurine and podophyllotoxin all produced suppression of labeling of this nuclear protein fraction. In most instances, this suppression was accompanied by decreased uptake of the tracer into other protein fractions. The data for 6-mercaptopurine and Myleran® most resembled those obtained with the aminouracil mustard in the apparent early specificity of effect on the acid-insoluble nuclear proteins without a marked suppression of amino acid incorporation into the cytoplasmic fractions of the tumor. The less selective agents in this respect included 5-fluorouracil, Leukeran®, HN2 and podophyllotoxin.

In other tissues, the effects were more varied than those found for the tumor. In the spleen, Leukeran® produced a marked inhibition of labeling of the nuclear proteins. 5-Fluorouracil suppressed labeling of the nuclear and cytoplasmic proteins of the spleen. On the other hand, marked effects on the labeling of the nuclear proteins were not produced in the spleen with either 6-mercaptopurine or Myleran® in these experiments.

In the liver, the aminouracil mustard produced essentially no change in the incorporation of L-arginine-U-^{14}C into cellular proteins. A similar result was found in these experiments for 6-mercaptopurine and for Leukeran®. However, 5-fluorouracil markedly suppressed the uptake of labeled arginine into all fractions of the liver and marked inhibition of labeling of the acid insoluble nuclear proteins and the microsomal proteins was found following injection of Myleran®.

The data presented in these studies suggest a relationship between antitumor activity and suppression of biosynthesis of the insoluble nuclear proteins. Fractionation of these proteins has been attempted with some success with the aid of DEAE columns.[13] Studies now in progress have been designed to overcome some of the problems of these chromatographic systems. Improvements have been made in technics for packing the columns and maintenance of constant flow rates for prolonged periods, which should provide improved resolution of the proteins. Investigations have recently been initiated on the lipoprotein components of the nuclear proteins.

* We are indebted to Dr. R. Bircher from Sandoz Pharmaceuticals for information on Podophyllotoxine Glucoside (SPG 827), as well as for a generous supply of the drug.

REFERENCES

1. H. Busch, S. M. Amer and W. L. Nyhan, *J. Pharmacol.* **127**, 195 (1959).
2. W. L. Nyhan and P. Pula, *Proc. Amer. Ass. Cancer Res.* **3**, 48 (1959).
3. H. Busch, J. Davis and D. C. Anderson, *Cancer Res.* **18**, 916 (1958).

4. H. BUSCH, W. STARBUCK and J. DAVIS, *Cancer Res.* **19**, 684 (1959).
5. C. HEIDELBERGER, L. GRIESBACH, B. J. MONTAG, D. MOOREN and O. CRUZ, *Cancer Res.* **18**, 305 (1958).
6. H. SKIPPER, J. R. THOMSON, G. B. ELION and G. H. HITCHINGS, *Cancer Res.* **14**, 294 (1954).
7. J. C. EVERETT, J. J. ROBERTS and W. C. J. ROSS, *J. Chem. Soc.* 2386 (1953).
8. D. A. LYTTLE and H. G. PETERING, *J. Nat. Cancer Inst.* **23**, 153 (1959).
9. W. C. J. ROSS, W. DAVIS, J. J. ROBERTS and J. L. EVERETT, *Ann. Rep. Brit. Emp. Cancer Campaign* **30**, 25 (1952).
10. G. M. TIMMIS, *Acta Un. Int. Cancrum* **10**, 181 (1954).
11. E. BOYLAND, J. W. CLEGG, P. C. KOLLER, E. RHODEN and O. H. WARWICK, *Brit. J. Cancer* **2**, 17 (1948).
12. H. LETTRE and H. BERGDOLT, *Z. Krebsforsch.* **59**, 68 (1953).
13. S. M. AMER and H. BUSCH, *Fed. Proc.* **19**, 338 (1960).
14. W. L. NYHAN, *Proc. Amer. Ass. Cancer Res.* **3**, 138 (1960).

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 135–150. Pergamon Press Ltd., Printed in Great Britain.

STUDIES ON THE ACTION OF SOME ANTI-INFLAMMATORY AGENTS IN INHIBITING THE BIOSYNTHESIS OF MUCOPOLYSACCHARIDE SULPHATES

M. W. WHITEHOUSE* and H. BOSTRÖM

Department of Metabolic Research, Wenner-Gren Institute, University of Stockholm
and the
Department of Medicine, Serafimerlasarettet, Karolinska Institutet, Stockholm, Sweden

(*Received* 2 *February* 1961)

Abstract—Salicylic acid and anti-inflammatory steroids inhibit the uptake of sulphate ions by cartilage and cornea *in vitro* and may further inhibit the utilization of the intracellular sulphate ions for the biosynthesis of sulphated mucopolysaccharides. These activities are ascribed to a common action of the drugs upon processes generating ATP within the tissues. The drug-sensitivity of connective tissues may increase with their age. The possible relationship between the biochemical action of these drugs and their therapeutic value as anti-inflammatory agents is discussed.

Some novel methods for the micro-estimation and quantitative isolation of polysaccharide sulphates using 6:9-diamino-2-ethoxy-acridine lactate (Rivanol) are described.

MUCOPOLYSACCHARIDE sulphates are characteristic components of the mesenchymal tissues in animals (connective tissue, tendon, cartilage etc.). Of especial importance are the chondroitin sulphates A and C and keratosulphate,† which are present in costal cartilage, the cornea and the nucleus pulposus.[1] Corneas contain a further polysaccharide, related in composition to the chondroitin sulphates but with a low content of ester sulphate (about 2 per cent), which has been designated "chondroitin".[2]

Certain drugs which are employed for the clinical management of rheumatic diseases, will inhibit the biosynthesis of mucopolysaccharide sulphates. These drugs include the salicylates, cortisone and many of its chemical analogues which are of value as anti-inflammatory agents.[3] We have sought to determine the precise action of these drugs upon the biosynthesis of cartilage and corneal polysaccharide sulphates.

Layton[4, 5] first noted a decrease in the incorporation of inorganic sulphate-^{35}S into various animal tissues *in vitro* and *in vivo*, following the administration of cortisone. He suggested that cortisone inhibited the biosynthesis of chondroitin sulphate in these tissues. This inhibitory action of cortisone has since been amply confirmed in numerous *in vivo* studies with the repeated observation of loss of metachromasia (associated with anionic polymers) and reduced incorporation of radiosulphate into connective tissues.[6] That cortisone acts directly upon connective tissue to inhibit the

* Permanent address: Department of Biochemistry, University of Oxford, Oxford, England.

† These polysaccharides have the composition: chondroitin sulphate A = polyglucuronido-N-acetylgalactosamine-4-sulphate; chondroitin sulphate C = polyglucuronido-N-acetylgalactosamine-6-sulphate; keratosulphate = polygalactosido-N-acetylglucosamine-6-sulphate.

biosynthesis of the mucopolysaccharide sulphates was demonstrated with cartilage slices *in vitro*.[7] Further experiments with cartilage-forming tissue cultures showed that not only cortisone but also a large number of related steroids with anti-inflammatory activity, inhibit chondrogenesis and the biosynthesis of chondroitin sulphate(s).[8] Analyses of the cartilage formed in tissue culture have suggested that it is the sulphation, and not the biosynthesis of the unsulphated polysaccharide, which is primarily affected by these steroids.[9, 10]

Salicylates have been shown to inhibit the incorporation of sulphate-^{35}S into cartilage polysaccharides *in vitro*.[11, 8]

We now present evidence that these steroids and the salicylates, though chemically unrelated, may have a common action in regulating the uptake and utilization of inorganic sulphate by cartilage and corneal slices.

EXPERIMENTAL METHODS

Materials for incubation

All incubations were conducted in freshly mixed modified Krebs–Ringer phosphate solution, pH 7·4. Magnesium chloride (0·11 M) was used in place of magnesium sulphate (0·15 M) to constitute the Krebs–Ringer solution. Preparation of steroid emulsions: Steroids were commercial products checked for authentic melting points. Aliquots of methanolic solutions of the steroids were transferred to test tubes containing a measured quantity of Tween 20 (polyoxyethylene sorbitan monolaurate, Atlas Powder Company, Wilmington, Delaware, U.S.A.) added as a solution in methanol. The methanol was removed from the mixture on warming in a water bath at 50 °C under a stream of air, leaving a solution of the steroid in droplets of Tween and a very small volume of methanol (<0·05 ml). Krebs–Ringer solution was added with vigorous shaking. Opalescent solutions were discarded. These emulsions usually contained two parts of Tween for each part of steroid. The efficiency of emulsification (and determination of the steroid in solution) was determined by measuring the optical density at 246 mμ after filtration, and comparing with the absorption of standard solutions of the steroids (Δ^4-3-ones) in ethanol.

Phenolic acids used were recrystallized samples (kindly donated by L. Reio), checked for homogeneity by paper chromatography and high-voltage paper electrophoresis. Solutions of the potassium salts (pH 7·4) were prepared by addition of potassium carbonate to suspensions of the acids in Krebs–Ringer solution. The precise concentration of the phenolic salt was determined spectrophotometrically at 246 mμ. Solutions of the dihydroxybenzoic acids were prepared afresh immediately before each incubation.

Circular cartilage slices (approx. 7 mm diameter, dry weight approx. 5 mg) were prepared as previously described[12, 13] from new-born calves and from cattle up to the age of 2 years, within 4 hr of slaughter. Circular "slices" of whole cornea (7 mm diameter, dry weight approx. 5·5 mg) were prepared from freshly excised calf and cattle corneas by "punching out" with a cork borer. The intact corneas and the individual slices were moistened continuously with Krebs–Ringer solution throughout this operation. Slices from a number of corneas were pooled in Krebs–Ringer solution, ten of these slices then being withdrawn at random for each incubation. Cartilage slices for any one series of experiments, were all prepared from one animal.

Vol. 7
1961

Incubations were conducted at 37 °C in air or oxygen with continuous slow shaking. Twenty cartilage slices or ten corneal slices were pre-incubated in a 50-ml flask with 9 ml of Krebs–Ringer solution containing steroids, phenols or other additions. The amount of Tween in this solution never exceeded 2 mg. After 15 min pre-incubation, sodium sulphate-^{35}S (5×10^5 counts/min) in 1 ml Krebs–Ringer solution was added to each flask. Incubation were continued for periods of up to 8 hr. When terminated, the medium was poured through a filter and the optical density at 246 mμ measured. Slices from each incubation were washed three times by decantation with chilled (4 °C) Krebs–Ringer solution, lightly blotted and stored in the moist state at 4 °C until taken for counting.

In each series of incubations, one flask contained slices, sulphate-^{35}S and sodium iodoacetate (5×10^{-3} M) and served as a control.

Measurement of incorporated radioactivity

(a) *First approximate (direct) assay.* Intact moist slices were plated in groups of four per plate on the special recessed plates, described for method II by Boström *et al.*[13] They were then dehydrated by standing in the refrigerator overnight and finally dried for 10 min at 100 °C. Corneal slices were grossly distorted unless dehydrated slowly in the refrigerator. The radioactivity of each plate with its dried slices, was measured directly with an end-window Geiger–Müller tube. The reproducibility between individual plates prepared with slices from any one incubation, was usually within the range 5–8 per cent.

(b) *Assay on digested slices.* Dried slices were digested with papain and the digests fractionated with Rivanol as described below. Aliquots (50 μl) were withdrawn from the whole digests, plated in quadruplicate on sand-blasted aluminium disks and counted to give a measure of the total incorporated ^{35}S (inorganic sulphate-^{35}S and polysaccharide sulphate-^{35}S). Aliquots (100-μl) from the soluble residue after precipitation of the polysaccharide sulphates, were likewise plated and counted to give a measure of the inorganic sulphate-^{35}S alone. Reproducibility between plates prepared from the one digest, was excellent ($\pm$3 per cent). Self-absorption was negligible though self-absorption curves were prepared.

Isolation of polysaccharide sulphates

Portions of cartilage and cornea (approximately 40 mg dry weight) in phosphate buffer (0·1 M, pH 6·8) were denatured by heating in a boiling water bath for 15 min to facilitate the subsequent digestion of collagen-like proteins (cf. Gardell[14]), then digested overnight with papain at 55 °C.[15] (The activated papain was prepared from one part of crude papain per fifteen parts dry weight of tissue, and filtered before use.) A small tissue residue resisted digestion (usually 5–10 per cent original dry weight) and was regularly discarded.

The pH of the digest was adjusted to 1·5 with hydrochloric acid and the precipitated papain and nucleic acids removed by centrifugation. Acidic polysaccharides, including chondroitin, were precipitated by the addition of 3% (w/v) Rivanol* (6:9-diamino-2-ethoxyacridine lactate monohydrate). Precipitation was complete

* Obtained from Bayer, Surbiton-on-Thames, England, under the name of "Ethodin". This compound is also variously designated: 2:5-diamino-7-ethoxyacridine lactate, "Acrinol", "Vucine", and "Acrolactine".

when the optical density at 410 mμ of the supernatant fraction, after centrifugation of the precipitate, exceeded 0·3. The precipitated polysaccharides were washed with distilled water and redissolved in 2 M ammonium formate. Diaminoethoxyacridinium ions were removed either by adsorption on alkali-washed Fuller's earth (Lloyd's reagent) or by extraction into *n*-butanol or phenoxyethanol.

Chemical analyses

Metachromatic polysaccharides were detected following high-voltage ionophoresis in carbonate buffers, pH 10·0, by immersion of dried paper strips in 0·15% (w/v) thionine in 65% (v/v) ethanol, acidified with approximately 1·0% (w/v) acetic acid. Excess stain was removed in running tap water.

Uronic acid content was determined colorimetrically with carbazole.[16] After addition of carbazole, tubes were returned to a boiling water bath for 10 min to hasten colour development. Formate ions considerably depressed colour formation. Addition of boric acid (cf. Gregory[17]) restored colour development towards normal. A final concentration of 1·5% (w/v) boric acid completely overcame the inhibition of 2 M ammonium formate.

Galactose content was determined colorimetrically by the cysteine–mannose–sulphuric acid reaction,[18] after ageing the developed coloration for 40 hr.

Total polysaccharide sulphates were determined turbidometrically on 10–100 μg samples contained in 2·5 ml of 0·8 M ammonium formate, after addition of Rivanol to a final concentration of 1 mg/ml. The optical densities of the turbidities formed were measured at 520 mμ, 20 min after the addition of Rivanol. Keratosulphate gave 70 per cent of the turbidity formed with the same amount of chondroitin sulphate. By this method, the total polysaccharide sulphates could be determined on much diluted samples of unfractionated cartilage digests. Corneal digests could not be so analysed directly for polysaccharide sulphates.

Reference materials

Chondroitin sulphate-^{35}S was isolated from calf cartilage incubated *in vitro* with sodium sulphate-^{35}S, by papain digestion and precipitation with cetyl pyridinium chloride.[15] We are indebted to Dr. J. E. Scott for this material. It was completely freed from inorganic sulphate-^{35}S by passage through a Dowex 1 column and finally precipitated with ethanol. This purified material contained 12 per cent keratosulphate (calculated from the galactose content).

Keratosulphate isolated from nucleus pulposus, contained less than 3 per cent galactosamine. Sodium chondroitin sulphate (14·2 per cent ester sulphate) isolated from calf tracheal cartilage, contained less than 1·5 per cent galactose. These polysaccharides were kindly donated by Mr. C. Antonopoulos and Dr. S. Gardell (Kemiska Laboratoriet, Serafimerlasarettet, Stockholm). Solutions of these polysaccharide sulphates were standardised by determination of their amino sugar content.[19]

EXPERIMENTAL RESULTS

Validity of the experimental methods

In order to assess the *total* sulphate-^{35}S (free and polysaccharide-bound) taken up by the incubated tissues, it was imperative to avoid losses of inorganic sulphate from the tissues after incubation. For this reason, the tissues were merely washed with

chilled Krebs–Ringer solution and counted after dehydration. Each series of incubations included at least one "contamination control" in which tissue slices were incubated with sodium sulphate-^{35}S with shaking either at 2 °C, or with added iodoacetate at 37 °C. Iodoacetate is a very potent inhibitor of the sulphate uptake by cartilage[12] and by corneas.[20] The extremely low radioactivity of cartilage slices incubated with iodoacetate (Table 1) was evidence for removal of all extraneous sul-

TABLE 1. UPTAKE OF SULPHATE-^{35}S BY CALF CARTILAGE AND CORNEA *in vitro*
(Incorporated ^{35}S measured by "direct assay". Final volume 10 ml. Cartilage incubated 2 hr, corneas incubated 5 hr.)

Addition	Temp. (°C)	Radioactivity per tissue slice Cartilage (counts/min)	Cornea (counts/min)
None	2	10	
None	37	542	1500
Iodoacetate	37	12	429
Methanol (25 μl)	37	561	1485
Methanol (50 μl)	37	530	1465
Methanol (100 μl)	37	470	
Tween 20 (2·5 mg)	37	587	1412
Tween 20 (10 mg)	37	343	

phate-^{35}S by the washing procedures employed. The association of sulphate-^{35}S with iodoacetate-poisoned corneal slices is probably a consequence of the considerable swelling and imbibition of water which accompanied incubation of these tissues. Further analyses revealed that on average, only 8 per cent of the total sulphate-^{35}S taken up by these poisoned slices was actually incorporated into the mucopolysaccharide sulphates. Table 1 also indicates the tolerance of the tissues to the amounts of Tween (200 μg/ml) and residual methanol ($<0{\cdot}5\%$ v/v) present in the steroid emulsions.

A quick accurate method was required for the quantitative separation of inorganic sulphate-^{35}S from the polysaccharide sulphates-^{35}S to allow their separate estimation, following digestion and solubilization of the cartilage and corneal slices with papain. Conventional precipitants of sulphated mucopolysaccharides (cationic detergents, streptomycin, hexamminocobaltic ions) were unsuitable for this purpose, as they all failed to *completely* precipitate the sub-milligram quantities of mucopolysaccharides liberated from digested corneal slices. Mucopolysaccharides constitute only about 2 per cent of the dry weight of cattle corneas.[2, 21] It was found that chondroitin sulphate and keratosulphate at concentrations of 20–500 μg/ml were quantitatively precipitated by Rivanol. The precipitated polysaccharide sulphates were readily soluble in 2 M ammonium formate, permitting suitable aliquots to be taken for counting. Table 2 records the results of some experiments testing the suitability of Rivanol for resolving artificial mixtures of inorganic sulphate and chondroitin sulphate or keratosulphate. Chondroitin sulphate (85 μg/ml) was quantitatively precipitated. At the same concentration, at least 92 per cent of the keratosulphate was precipitated. This was considered satisfactory in view of the difficulty in precipitating keratosulphate at all with cationic detergents.[15] Inorganic sulphate was not co-precipitated with the polysaccharides.

Effect of salicylic acid and related compounds

Potassium salicylate added at 5×10^{-3} M or in higher concentrations, considerably inhibited the sulphate uptake by fresh calf cartilage and corneal slices (Table 3). Lower concentrations were not inhibitory and in many experiments, even stimulated the sulphate uptake.

TABLE 2. SEPARATION OF SULPHATE IONS AND POLYSACCHARIDE SULPHATES WITH RIVANOL

(ChS = chondroitin sulphate, measured as glucuronic acid.
KS = keratosulphate, measured as galactose.
Precipitation in volume = 1·2 ml, pH 1·5; Rivanol = 0·1 ml, 1 per cent (w/v).
Precipitates redissolved in 1·2 ml 2 M ammonium formate.)

Composition of mixture	Rivanol added	Residual supernatant O.D.*	Residual supernatant (counts/min)	Redissolved ppt. O.D.*	Redissolved ppt. (counts/min)†
100 μg.KS + $^{35}SO_4^{=}$	−	0·112	12,050	—	—
	+	0·010	12,070	0·103	10
20 μg.KS + $^{35}SO_4^{=}$	−	0·022	5910	—	—
	+	0·002	5895	0·023	10
100 μg.ChS + $^{35}SO_4^{=}$	−	0·210	7555	—	—
	+	0·005	7465	0·218	17
100 μg.ChS-^{35}S	−		1500		—
	+		17		1490
10 μg.ChS-^{35}S	−		153		—
	+		10		140
Digest‡ + 50 μg.ChS-^{35}S	−		724		—
	+		4		742

* O.D. = optical density at 535 mμ (uronic acid) or 600 mμ (galactose).
† Uncorrected for self-absorption.
‡ Papain-digested cartilage.

Magnesium ions are probably essential for mucopolysaccharide biosynthesis and sulphation. Salicylic acid forms a complex with magnesium ions, albeit at rather an alkaline pH.[22] Conducting incubations in the absence of added magnesium ions or with the addition of a more potent magnesium-complexing agent, versene (ethylene-diaminetetra-acetate), failed to mimic the inhibitory action of potassium salicylate (Table 3).

A number of related aromatic compounds were tested for their effect on the sulphate uptake by cartilage slices. Only *o*-pyrocatechuate, L-thyroxine and 2:4-dinitrophenol were inhibitory (Table 4).

Effect of hydrocortisone and related compounds

Hydrocortisone and cortisone at 10^{-4} M inhibited the sulphate uptake by cartilage slices, their 21-acetates being much less inhibitory. Deoxycorticosterone was also a much weaker inhibitor. The degree of inhibition appeared to increase with increasing age of the tissues. Table 5 records the results obtained when equal volumes of the same steroid emulsion were incubated with slices of cartilage and with corneas from young and old animals.

Because it is difficult to obtain the exact age of cattle slaughtered, some attempts were made to define the age of the incubated tissues by biochemical parameters. The

Vol. 7
1961

proportion of keratosulphate in human, chick and invertebrate cartilage increases with age.[23, 24] Therefore the ratio of the uronic acid to the galactose contents of the Rivanol-precipitated mucopolysaccharides, was determined after digesting slices of young calf and cow cartilage and corneas with papain. A decrease in this ratio represents an increase in the proportion of keratosulphate relative to the sum of the

TABLE 3. EFFECT OF POTASSIUM SALICYLATE, VERSENE AND MAGNESIUM IONS ON SULPHATE-^{35}S INCORPORATION BY CALF CARTILAGE AND CORNEAL SLICES

(Incorporated ^{35}S = radioactivity per slice; for cartilage, determined by direct assay; for cornea, after papain digestion. Incubation period, 6 hr.)

Addition	Cartilage		Cornea	
	Incorporated ^{35}S (counts/min)	% Inhibition	Incorporated ^{35}S (counts/min)	% Inhibition*
None	1800	—	2816	—
Salicylate				
5×10^{-2} M	96	95		
5×10^{-3} M	715	60	1603	43
5×10^{-4} M	1920	−7†	2347	17
5×10^{-5} M	2033	−13†	3157	−11†
Versene				
10^{-3} M	2038	−13†		
10^{-5} M	2040	−13†		
Without Mg^{2+}	2400	−33†		
Without Ca^{2+}	1960	−9†		
Without both Mg^{2+} + Ca^{2+}	2485	−37†		
Iodoacetate ($2{\cdot}5 \times 10^{-3}$ M)	25	(98)	1385	(51)

* Uncorrected for uptake by iodoacetate-poisoned slices.
† Actually a stimulation.

TABLE 4. EFFECT OF SALICYLIC ACID AND RELATED PHENOLS UPON SULPHATE-^{35}S INCORPORATION INTO CARTILAGE SLICES

(Incorporated ^{35}S measured by direct assay. Aromatic acids added as potassium salts. Incubation period, 6 hr.)

Addition	Concentration	Incorporated ^{35}S/slice (counts/min)	% Inhibition
None		1075	—
Salicylate	5×10^{-3} M	545	49
o-Pyrocatechuate (2:3-dihydroxybenzoate)	5×10^{-3} M	707	34
Gentisate (2:5-dihydroxybenzoate)	5×10^{-3} M	1055	2
γ-Resorcylate (2:6-dihydroxybenzoate)	5×10^{-3} M	1005	7
2:4-Dinitrophenol	5×10^{-4} M	225	79
	5×10^{-5} M	561	48
L-Thyroxine	$2{\cdot}5 \times 10^{-4}$ M	734	31
	$2{\cdot}5 \times 10^{-5}$ M	1011	6
Phenol	5×10^{-3} M	1034	4
Benzoate	5×10^{-3} M	1067	0

chondroitin sulphates A and C and chondroitin. Some other parameters related to the biological age of the tissues were also noted: water content of the fresh tissues and the relative activities of the slices tissues in taking up inorganic sulphate-^{35}S from the medium in the absence of drugs.

The concentration of the steroids (measured as Δ^4-3-ones) present in the medium after 6 hr incubation was always found to be at least 93 per cent (93–99·5 per cent)

TABLE 5. EFFECT OF AGE ON DRUG-SENSITIVITY OF SULPHATE-^{35}S UPTAKE BY CATTLE CARTILAGE AND CORNEAS *in vitro*

(Incubation period, 6 hr. Additions: steroids, 10^{-4} M; salicylate, 5×10^{-3} M; iodoacetate, 5×10^{-3} M.)

Addition	% Inhibition $^{35}SO_4^{2-}$ uptake by Cartilage		Cornea	
	Calf (%)	Cow (%)	Calf (%)	Cow (%)
Hydrocortisone				
alcohol	23	70	10	54
acetate	4	4		
Cortisone				
alcohol	29	47		
acetate	0	5		
Deoxycorticosterone	2	3		
Sailcylic acid	64	72	56	62
Iodoacetate	98	98	71	78
Tissue parameters:				
Uronic acid: galactose	2·2	1·5	0·9	0·8
Dry: wet weight	23·0%	29·6%	20·1%	21·4%
^{35}S uptake by control (counts/min per slice)	2550	480	1500	840

of the initial concentration. There was evidently little or no metabolism (biological reduction) of the inhibitory steroids by the tissues during the incubation period.

No evidence was found for any drug-stimulated desulphation and depolymerization of the mucopolysaccharide sulphates in cartilage, as might be detected by reduction in the turbidities formed with Rivanol. The total polysaccharide sulphate content of slices incubated with hydrocortisone for periods up to 8 hr, was not significantly less than that of slices which had not been incubated at all or which had been incubated for the same period without hydrocortisone. There being no detectable change in the amount of mucopolysaccharide sulphates within the tissue, the only explanation for the observed reduction in the polysaccharide sulphate-^{35}S fraction as a result of drug action, is that incorporation of inorganic sulphate-^{35}S into the polysaccharides was inhibited.

Inorganic sulphate-^{35}S within the cartilage and cornea slices

The inorganic sulphate-^{35}S within the tissues is rapidly incorporated into the polysaccharide sulphates. More than 80 per cent of the total ^{35}S present within cartilage slices incubated for only 5 min with sodium sulphate-^{35}S, was precipitated with Rivanol. The proportion of polysaccharide sulphate-^{35}S increased to approximately 92 per cent of the total ^{35}S in the tissues after 30 min incubation, and to 95 per cent after 6 hr incubation.

Vol. 7
1961

The following experiment showed that the radioactivity of papain-digests remaining in solution after the Rivanol precipitation, was indeed intracellular inorganic sulphate-^{35}S (or very labile organic sulphate-^{35}S) and not small traces of unprecipitated polysaccharide sulphate-^{35}S. Aliquots of papain digests from a normal 6 hr incubation and from its iodoacetate-poisoned control were submitted to paper ionophoresis. The zones ahead of the foremost metachromatic material (chondroitin sulphates) were eluted. This area represents inorganic sulphate. Direct comparison of the radioactivity eluted from these zones showed that the slices from the normal incubation contained much more free (or labile) sulphate-^{35}S (270 counts/min) than the iodoacetate-poisoned control (48 counts/min).

The proportion of inorganic sulphate-^{35}S relative to the total sulphate-^{35}S (inorganic and polysaccharide-bound) within the tissues always increased when the slices had been incubated with the anti-inflammatory steroids or with uncoupling agents (Table 6). This finding supports the previous inference that under the action of the drugs,

TABLE 6. DISTRIBUTION OF INCORPORATED ^{35}S BETWEEN INORGANIC SULPHATE AND POLYSACCHARIDE SULPHATES IN CALF CARTILAGE AND CORNEA

(Incubation period, 5·5 hr.)

Addition	Cartilage (10 slices)			Cornea (8 slices)		
	Total ^{35}S (counts/min)	$^{35}SO_4^{2-}$ (counts/min)	$^{35}SO_4^{2-}$ (%)	Total ^{35}S (counts/min)	$^{35}SO_4^{2-}$ (counts/min)	$^{35}SO_4^{2-}$ (%)
None	59,500	2400	4·0	25,820	11,200	43
Salicylate (5×10^{-3} M)	28,400	1660	5·7	16,030	10,000	62
Dinitrophenol (5×10^{-4} M)	8300	1030	12·4			
Tween only	41,400	1800	4·3	26,280	11,300	43
Hydrocortisone (2×10^{-4} M)	24,100	1350	5·6	25,120	12,600	50
Cortisone (2×10^{-4} M)	21,100	1160	5·5			
Iodoacetate (5×10^{-3} M)				13,850	12,700	92

there is diminished incorporation of the inorganic sulphate-^{35}S, present within the tissues, into the mucopolysaccharide sulphates. However, the actual amount of inorganic sulphate-^{35}S in the drug-incubated tissues was always less than that in the corresponding controls.

If only the utilization of the intracellular sulphate had been inhibited, the labelling of the intracellular sulphate pool should have increased. There are two possible explanations for the observed reduction in the intracellular inorganic sulphate-^{35}S under the influence of the drugs. One explanation requires that in response to the drugs, the intracellular inorganic sulphate pool is augmented by non-radioactive sulphate so diluting the inorganic sulphate-^{35}S, and the excess sulphate is then excreted into the medium. This non-radioactive sulphate could only arise from within the tissue as the sulphate in the medium was radioactive. There was, however, no evidence of a drug-induced degradation of the polysaccharide sulphates, which might have liberated inorganic sulphate within the tissues. The other, simpler and more acceptable interpretation of the diminished labelling of intracellular sulphate, is that the drugs

inhibited the transport of sulphate ions across the permeability barriers between the medium and the tissues. This cannot be the only action of the drugs, for we always observed an increase in the *proportion* of inorganic sulphate-^{35}S in the drug-treated tissues, even though the actual *amount* was less than the inorganic sulphate-^{35}S present in tissues which had been similarly incubated without the addition of the drugs.

DISCUSSION

Connective tissue is rather sparsely populated with active and proliferating cells, by comparison with other body tissues. The rates of respiration and intermediary metabolism of the connective tissues are rather low and not easily measured experimentally. These tissues are relatively rich in mucopolysaccharide sulphates. The ready utilization of inorganic sulphate for their biosynthesis does afford one criterion of biochemical activity of the mesenchymal cells, readily measured with the availability of radioactive sulphate (cf. Aurell *et al.*[20]).

The inhibition of mucopolysaccharide sulphate biosynthesis by salicylates and by cortisone and its congeners, may actually be of little therapeutic value. It does, however, afford a useful experimental index with which to study whether or not these chemically dissimilar drugs have any common biochemical action, which might explain their similar pharmacological action in suppressing tissue inflammation.

Cartilage and cornea were chosen for this study since they are readily accessible and it is possible to prepare slices therefrom which do not vary greatly in activity between consecutive experiments. In cartilage the chondroitin sulphates predominate but the proportion of keratosulphate increases with age.[23, 24] In cornea, keratosulphate is the predominant polysaccharide.[2, 21] Thus, the cornea in some respects resembles "elderly" cartilage. In cartilage, mucopolysaccharide sulphates are major constituents (30–40 per cent dry weight); in cornea, they are only minor constituents (2–3 per cent dry weight). The two tissues are therefore somewhat complimentary. Incorporation of sulphate ions into the mucopolysaccharide sulphates of cornea has been described by a number of workers[20, 25, 26] but the possible effects of drugs and hormones upon corneal metabolism has hardly been investigated to date. Sulphate metabolism in cartilage has been studied more intensively.[27, 28]

We now have evidence that in both cornea and cartilage, salicylates and the anti-inflammatory steroids inhibit the incorporation of extra-cellular inorganic sulphate into the mucopolysaccharide sulphates of the tissues, by diminishing the uptake of sulphate ions by the tissues and also by partially inhibiting the incorporation of the intracellular inorganic sulphate into the polysaccharide sulphates (Fig. 1).

The specific biochemical action of salicylic acid

As in cartilage and cornea, the incorporation of sulphate ions into the polysaccharide sulphates of granulation tissue is effectively inhibited by salicylic acid.[29] Though granulation tissue and cornea both possess enzyme systems for sulphating phenol, neither of these tissues would sulphate salicylic acid and its salts.[29, 30] By contrast, other compounds which are biologically sulphated in the cornea and elsewhere in the animal body,[31] e.g. ethanol or phenol itself, do not inhibit incorporation of sulphate ions into the mucopolysaccharide sulphates *in vitro* when administered at the same concentrations (10^{-3} M) at which salicylic acid is an effective inhibitor. It therefore

Vol. 7
1961

seems quite improbable that salicylic acid exerts its inhibitory action by acting as an alternative sulphate acceptor, being itself sulphated, thereby diverting the supply of active sulphate (PAPS) from the normal sulphate acceptors (oligo/polysaccharides). Nor can the action of salicylic acid be simply explained by the complexing of magnesium ions, as our experiments have shown.

Amongst the many phenolic compounds tested in the present and in previous experiments,[11] the only powerful inhibitors of sulphate incorporation into cartilage

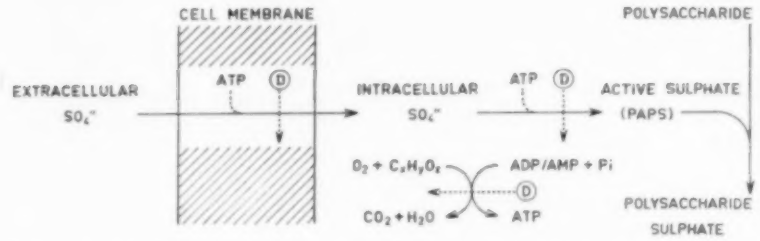


FIG. 1. Scheme depicting the probable pathway of sulphate metabolism and sites of drug inhibition (D).

were L-thyroxine and 2:4-dinitrophenol. These are both potent uncouplers of oxidative phosphorylation in mitochondria isolated from various animal tissues.[32] Salicylic acid is known to uncouple oxidative phosphorylation in rat liver and heart muscle mitochondria.[33–37] It is instructive to compare the relative potencies of dinitrophenol, thyroxine and salicylic acid in this respect. Table 7 compares the concentrations of

TABLE 7. COMPARISON OF SALICYLIC ACID WITH DINITROPHENOL AND THYROXINE
(See text for references)

Compound	Equi-potent concentrations for action on		
	Cartilage	Heart mitochondria	Yeast
Salicylic acid	5×10^{-3} M	$2{\cdot}5 \times 10^{-3}$ M	8×10^{-3} M
L-Thyroxine	4×10^{-4} M	5×10^{-4} M	—
2:4-Dinitrophenol	5×10^{-5} M	$< 10^{-4}$ M	10^{-4} M

these three compounds which inhibit sulphate incorporation into calf cartilage by 50 per cent and which produce the same degree of uncoupling of oxidative phosphorylation in heart mitochondria[37] and in yeast,[38] as measured by the consequent stimulation of respiration. It is yet more instructive to compare the relative activities of salicylic acid and some of its analogs, as inhibitors of sulphate incorporation into cartilage slices and as inhibitors of oxidative phosphorylation in rat liver mitochondria[33] (Table 8). The agreement between the relative potencies of these analogs acting on the two systems, is remarkable. The only exceptions (with methyl salicylate and acetyl salicylate) probably reflect the different rates of hydrolysis of these particular derivatives in cartilage and in liver mitochondrial preparations.

The stimulation of sulphate uptake by low concentrations of salicylate (5×10^{-5} M) was repeatedly observed in these experiments. It may represent chelation of some inhibitory metal ion, or possibly a "tightening" of the coupling between oxidation and phosphorylation. Such an apparent tightening has been observed with rat liver mitochondria at lower concentrations of nicotinic acid (10^{-3} M), whilst higher concentrations of nicotinic acid ($1{\cdot}5 \times 10^{-2}$ M) uncoupled oxidative phosphorylation. (Whitehouse, unpublished experiments.)

Table 8. Relative activities of salicylic acid and its analogues

Compound	Relative inhibition of Sulphate uptake by cartilage I* (%)	II† (%)	Oxidative phosphorylation in rat liver mitochondria‡ (%)
Salicylic acid	28	49	96
Methyl salicylate	15		74
Acetyl salicylate	16		72
Salicylamide	8		11
p-Aminosalicylic acid	1		3
2:3-Dihydroxybenzoic acid (*o*-pyrocatechuic acid)		34	55
2:4-Dihydroxybenzoic acid (*β*-resorcylic acid)	3		9
2:5-Dihydroxybenzoic acid (gentisic acid)	2	2	7
2:6-Dihydroxybenzoic acid (*γ*-resorcylic acid)		7	10
Benzoic acid		0	0

* From Boström and Månsson[11], with concentrations 4×10^{-2} M.
† Present experiments, with concentrations 5×10^{-2} M.
‡ From Brody[20], with concentrations 2×10^{-2} M.

The action of the steroids

There appears to be a correlation between the activity of the various steroids studied, in inhibiting sulphate uptake by mesenchymal tissues and their potency as anti-inflammatory agents.[8, 10] In both respects, cortisone and hydrocortisone (cortisol) are the most active of the natural steroids secreted by the adrenal cortex. Their respective esters and other steroid hormones (desoxycorticosterone, progesterone, etc.) were either much less active or completely inactive as inhibitors of sulphate metabolism in connective tissues. We have always found that cortisone and hydrocortisone have similar activity on three *in vitro* systems, viz. chick embryonic cartilage,[10] cattle cartilage and cattle cornea. There are, however, reports in the literature which suggest that hydrocortisone has no action on cartilage metabolism, in contradistinction to cortisone. Thus Clark and Umbreit[39] have described the inhibition of sulphate incorporation into rat xiphoid cartilage *in vitro* by cortisone and cortisone acetate and a stimulation of the sulphate uptake by unspecified concentrations of hydrocortisone and hydrocortisone acetate. For these experiments, the steroids were "dissolved" directly in the incubation medium; a procedure which we have found to be unreproducible and unsatisfactory. Another report[40] has noted differences between the action of cortisone acetate and hydrocortisone acetate on cartilage metabolism

Vol. 7
1961

in vivo. If indeed only the steroid alcohols are pharmacologically active, as suggested by *in vitro* studies, such discrepancies may reflect different rates of hydrolysis of cortisone acetate and hydrocortisone acetate in the whole animal, or different rates of inactivation of the steroid alcohols formed from the acetates *in vivo*.

A number of independent reports[41–46] have described the inhibition of mitochondrial oxidation by hydrocortisone and other steroid hormones when added at concentrations of the order of 10^{-4} M. There is common agreement that succinate oxidation is not inhibited and that the oxidation of other substrates mediated by DPN and the oxidation of DPNH itself is steroid-sensitive. Gallagher[46] reported that the inhibitory action of hydrocortisone on rat liver mitochondria is reversed by adding back necessary cofactors and has suggested that hydrocortisone may act as a regulator of metabolism by governing the selective semipermeability of the mitochondrial membranes. In all instances, addition of the steroids primarily affected the rates of oxidation of the various substrates (excepting succinate) by the mitochondria and not oxidative phosphorylation. Cortisone may possibly uncouple oxidative phosphorylation in liver mitochondria.[47]

Grossfeld[48] has reported some data which suggest the relevance of these observations upon isolated mitochondria, to the action of the steroids upon connective tissue. He found that hydrocortisone (3×10^{-4} M) strongly inhibited the respiration of fibroblasts in tissue culture. Fibroblasts are mesenchymal cells of the type found widely distributed throughout connective tissues. It is therefore interesting to note that desoxycorticosterone was much less active than hydrocortisone in inhibiting both fibroblast respiration[48] and sulphate metabolism in cartilage, and is almost devoid of anti-inflammatory activity.

The *net* effect of this diminished respiration (in response to the steroids) would be the same as if direct uncoupling of oxidative phosphorylation had occurred (as with the salicylates), with reduction in the amounts of ATP generated by mitochondrial oxidation.

Relationship between the biochemical and the therapeutic actions of cortisone and salicylates

Certain tissues derived from the primitive mesenchyme including connective tissue, synovia, vascular tissue and muscle are prominently involved in the so-called "collagen diseases". Inflammation of these tissues, caused by a variety of noxious agents, is strikingly modified by several drugs including the salicylates and congeners of cortisone. These particular drugs also modify the clinical course of a group of disorders of mesenchymal tissue with differing clinical manifestations and perhaps caused by different agents. The biological reactivity of the mesenchymal tissues to these agents (inflammation, tissue swelling, etc.) is depressed by the drugs. It seems reasonable to suppose that the drugs control the tissue reactivity at some fundamental biochemical level, common to a variety of animal species and to the diverse reactions elicited by a variety of injurious agents.

Adams and Cobb[49] drew attention to the possible relationship between anti-inflammatory activity and ability to uncouple oxidative phosphorylation. They noted, however, that 2:3-dihydroxybenzoic acid (*o*-pyrocatechuic acid) and dinitrophenol, though active as uncoupling agents, were without anti-inflammatory activity in the

erythrema inhibition assay. This assay may not truly represent the therapeutic value of the compound since pyrocatechuic acid is a useful anti-rheumatic drug.[50] The concentrations at which salicylic acid is a potent inhibitor of both oxidative phosphorylation and sulphate metabolism in cartilage ($2–5 \times 10^{-3}$ M) do correspond closely with the plasma salicylate levels of 20–30 mg % (ca. 2×10^{-3} M) which must be maintained for effective therapy in rheumatic fever.[51] Moses and Smith[52] presented data that seem to preclude any common action of anti-inflammatory steroids and of salicylic acid upon carbohydrate metabolism in liver and brain preparations. It is of course uncertain how relevant their observations may be to the action of the drugs on the intact connective tissues.

Though acting by different mechanisms, the anti-inflammatory steroids and salicylates may yet have a common effect by reducing ATP synthesis, thereby partially inhibiting a multitude of endergonic processes in the tissues. As a consequence, not only will biosynthetic mechanisms be inhibited but so will the ATP-dependent movements of certain metabolites, ions and water between the extracellular fluid and the interior of the tissues. These active transport mechanisms are often very sensitive to changes in the concentration of ATP within the cells.[53, 54] Smith[51] has discussed possible benefits of alterations in these active transport mechanisms in countering normal tissue swelling and inflammation elicited by noxious agents.

Our own observations indicate that both the transport of sulphate ions across the cell membranes and the (ATP-dependent) utilization of intracellular sulphate ions for sulphation of the mucopolysaccharides, were inhibited by the uncoupling agents, e.g. salicylic acid, and by the anti-inflammatory steroids. We can only explain this double action of these chemically dissimilar drugs in terms of an inhibition of the biosynthesis of ATP within the connective tissues. That the sulphation rather than the biosynthesis, of the (unsulphated) mucopolysaccharides should primarily be affected by the drugs[16] may reflect the relative availability of sulphate ions and carbohydrate precursors within the tissues subjected to the action of the drugs. Salicylates may actually increase glucose uptake by some tissues[55] and the anti-inflammatory steroids all promote gluconeogenesis. Thus the drugs may not diminish the supply of carbohydrate precursors for mucopolysaccharide biosynthesis. Any action of these drugs curtailing the active transport of sulphate ions from the extracellular fluid into the mesenchymal tissues, through limiting the supply of ATP, might become a determinant factor in controlling the eventual degree of sulphation of the biosynthesized mucopolysaccharides. Grossfeld, Meyer and their co-workers[56] have found that sulphated polysaccharides formed by skin, subcutaneous tissue and embryonic bone in tissue cultures, may be "undersulphated" and concluded that sulphation of the mucopolysaccharide molecules may indeed proceed independently of their biosynthesis.

The efficacy of some anti-inflammatory drugs may perhaps depend on the age of the target tissues. Disorders of connective tissues usually increase in severity with age and might be considered "diseases of ageing". It was therefore interesting to find that the sensitivity of elderly cartilage and cornea to cortisone and hydrocortisone was greater than that of more juvenile tissues. Whether this increase in drug-sensitivity with age rests with the declining activity of mitochondria within the tissues or is due to other physicochemical changes in the tissues with ageing (e.g. increase in keratosulphate, decrease in water content) is problematical. It should be noted that the

Vol. 7
1961

relative inhibition of sulphate metabolism in cartilage and the cornea by salicylic acid was rather less age-dependent.

Acknowledgements—We thank Prof. G. Biörck for his interest in this work. It is also a pleasure to acknowledge the assistance and advice we have received from Messrs. H. Johnsson and A. Vestermark, Drs. S. Gardell and L. Rodén. One of us (M.W.W.) is greatly indebted to the Empire Rheumatism Council, Great Britain, for the award of a Geigy travelling Fellowship. This investigation was supported by grants from the Helen Hay Whitney Foundation, New York, the National Institutes of Health (no. A.2568 M & N), Bethesda, and from King Gustaf the Fifth's Eightieth Birthday Fund.

REFERENCES

1. K. Meyer *Struktur und Stoffwechsel des Bindegewebes* (Edited by W. H. Hauss and H. Losse) p. 12. Georg Thieme Verlag, Stuttgart (1960).
2. K. Meyer, A. Linker, E. A. Davidson and B. Weissmann, *J. Biol. Chem.* **205**, 611 (1953).
3. J. Fried and A. Borman, *Vitam. and Horm.* **16**, 303 (1958).
4. L. L. Layton, *Arch. Biochem. Biophys.* **32**, 224 (1951).
5. L. L. Layton, *Proc. Soc. Exp. Biol., N.Y.* **76**, 596 (1951).
6. G. Asboe-Hansen, *Physiol. Rev.* **38**, 446 (1958).
7. H. Boström and E. Odeblad, *Ark. Kemi* **6**, 39 (1953).
8. J. W. Lash and M. W. Whitehouse, *Lab. Invest.* **10**, 388 (1961).
9. M. W. Whitehouse and J. W. Lash, *Acta Endocrinol.* Suppl. No. 51, 957 (1960).
10. M. W. Whitehouse and J. W. Lash, *Nature, Lond.* **189**, 37 (1961).
11. H. Boström and B. Månsson, *J. Pharm., Lond.* **7**, 185 (1955).
12. H. Boström and B. Månsson, *Ark. Kemi* **6**, 23 (1953).
13. H. Boström, E. Jorpes, B. Månsson, L. Rodén and A. Vestermark, *Ark. Kemi* **8**, 469 (1955).
14. S. Gardell, *Ark. Kemi* **4**, 449 (1952).
15. J. E. Scott, *Methods of Biochemical Analysis* (Edited by D. Glick) Vol. VIII, p. 145. Interscience, New York and London (1960).
16. Z. Dische, *J. Biol. Chem.* **167**, 189 (1947).
17. J. D. Gregory, *Arch. Biochem. Biophys.* **89**, 157 (1960).
18. Z. Dische, L. B. Shettles and M. Osnos, *Arch. Biochem. Biophys.* **22**, 167 (1949).
19. S. Gardell, *Methods of Biochemical Analysis* (Edited by D. Glick) Vol. VI, p. 289. Interscience, New York and London (1960).
20. G. Aurell, C-H. Dohlman and L. Rodén, *Acta Opthalmol.* **34**, 281 (1956).
21. J. Polatnick, A. J. la Tessa and H. M. Katzin, *Biochem. Biophys. Acta* **26**, 361 (1957).
22. C. A. Zittle, L. Pepper and E. W. Bingham, *Arch. Biochem. Biophys.* **88**, 94 (1960).
23. K. Meyer and D. Kaplan, *Nature, Lond.* **183**, 1267 (1959).
24. J. W. Lash and M. W. Whitehouse, *Arch. Biochem. Biophys.* **90**, 159 (1960).
25. B. Wortman and J. L. Strominger, *Amer. J. Opthalmol.* **44**, 291 (1957).
26. M. Suda, *Jap. J. Opthalmol.* **2**, 172 (1958).
27. H. Boström, *Struktur und Stoffwechsel des Bindegewebes* (Edited by W. H. Haus and H. Losse) p. 20. Georg Thieme Verlag, Stuttgart (1960).
28. H. Boström and L. Rodén, *The Chemistry and Biology of Compounds Containing Aminosugars* (Edited by R. W. Jeanloz and E. A. Balazs). Academic Press, New York (1961).
29. G. Loewi and P. W. Kent, *Biochem. J.* **65**, 550 (1957).
30. B. Wortman, *Amer. J. Physiol.* **198**, 779 (1960).
31. H. Boström and A. Vestermark, *Biochem. Pharmacol.* **6**, 72.
32. T. M. Brody, *Parmacol. Revs.* **7**, 335 (1955).
33. T. M. Brody, *J. Pharmacol. Therap.* **117**, 39 (1956).
34. I. Bosund, *Acta Chem. Scand.* **11**, 541 (1957).
35. R. Penniall, *Biochem. Biophys. Acta* **30**, 247 (1958).
36. S. W. Jeffrey and M. J. H. Smith, *Biochem. J.* **72**, 462 (1959).
37. L. Packer, F. K. Austen and E. C. Knoblock, *Proc. Soc. Exp. Biol., N.Y.* **100**, 239 (1959).
38. V. Moses and M. J. H. Smith, *Biochem. J.* **74**, 18P (1960).
39. I. Clark and W. W. Umbreit, *Proc. Soc. Exp. Biol., N.Y.* **86**, 558 (1954).
40. R. Lotmar, *Experientia* **16**, 303 (1960).

41. M. F. BIRABEN, R. LOWY and J. TRÉMOLIÈRES, *C.R. Soc. Biol., Paris* **92**, 936 (1958).
42. R. LOWY, R. DERACHE and J. TRÉMOLIÈRES, *Bull. Soc. Chim. Biol., Paris* **40**, 127, 137, 151 (1958).
43. W. WERNITSCH, H. J. HÜBENER and S. CLOEREN, *Hoppe-Seyl. Z.* **313**, 12 (1958).
44. P. K. JENSEN, *Nature, Lond.* **184**, 451 (1959).
45. K. L. YIELDING and G. M. TOMKINS, *Proc. Nat. Acad. Sci., Wash.* **45**, 1730 (1959).
46. C. H. GALLAGHER, *Biochem. J.* **74**, 38 (1960).
47. W. KERPPOLA, *Endocrinology* **67**, 92 (1960).
48. H. GROSSFELD, *Endocrinology* **65**, 777 (1959).
49. S. S. ADAMS and R. COBB, *Nature, Lond.* **181**, 773 (1958).
50. N. E. CLARKE, C. N. CLARKE and R. E. MOSHER, *Amer. J. Med. Sci.* **235**, 7 (1958).
51. M. J. H. SMITH, *J. Pharm., Lond.* **11**, 705 (1959).
52. V. MOSES and M. J. H. SMITH, *Biochem. J.* **78**, 424 (1961).
53. B. ANDERSON and H. H. USSING, *Comparative Biochemistry* (Edited by M. FLORKIN and H. S. MASON) Vol. II, p. 371. Academic Press, New York and London (1960).
54. R. WHITTAM, *Ann. Rep. Progr. Chem.* **57**. In press.
55. P. J. RANDLE and G. H. SMITH, *Biochem. J.* **70**, 490 (1958).
56. H. GROSSFELD, K. MEYER, G. GODMAN and A. LINKER, *J. Biochem. Biophys. Cytol.* **3**, 391 (1957).

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 151–153. Pergamon Press Ltd., Printed in Great Britain.

INHIBITION OF RAT SERUM GLUTAMIC-PYRUVIC TRANSAMINASE *IN VITRO* BY CONGENERS OF SALICYLATE

R. A. STEGGLE, A. K. HUGGINS and M. J. H. SMITH

Department of Chemical Pathology,
King's College Hospital Medical School, Denmark Hill, London, S.E.5

(*Received* 29 *December* 1960)

Abstract—The relation between chemical structure and inhibitory activity against rat serum glutamic-pyruvic transaminase was studied in congeners of salicylate. The presence of a phenolic hydroxyl in the *ortho*-position to a carboxyl group was found to be a necessary structural requirement for inhibitory activity except that a thiol group may replace the phenolic hydroxyl.

INTRODUCTION

SALICYLATE produces effects on the incorporation of radioactivity from labelled glucose and acetate into the soluble metabolic intermediates of isolated tissues which are explicable in terms of an uncoupling action on oxidative phosphorylation processes.[1] However, using [3-^{14}C]pyruvate as the labelled substrate, salicylate, but not the more powerful uncoupling reagent 2:4-dinitrophenol, produced an increased accumulation of radioactivity in glutamic acid.[2] This result suggested that salicylate exerted an effect on glutamic acid metabolism distinct from its uncoupling action and it was found that salicylate inhibited rat serum glutamic–pyruvic transaminase activity *in vitro*.[2] The present paper is concerned with a study of the relation between chemical structure and the inhibition of the transaminase activity in congeners of salicylate.

EXPERIMENTAL

Materials

The salicylate congeners were obtained commercially and recrystallized from suitable solvents until their melting points remained constant. They were dissolved in 0·1 M KH_2PO_4 solution at pH 7·4 to give solutions which after admixture with the other constituents of the reaction mixture produced a final concentration of 5 mM. Some of the congeners were too insoluble for this final concentration to be attained and were used as saturated solutions at 37 °C.

Measurement of transaminase activity

Glutamic–pyruvic transaminase activity was measured in pooled serum obtained from adult male rats of the Wistar strain by the method of Reitman and Frankel[3]. The determinations were made with a Technicon Autoanalyser (Technicon Instrument Co. Ltd., London) which consists essentially of a flow system in which the constituents of the reaction mixture (Serum, congener solution, substrate and colour reagent) are added successively and the final colour, developed after incubation,

is measured by a recording absorptiometer thus enabling continuous analyses to be performed. A minimum of four determinations was made for each congener and corresponding control mixtures, in which the congener solutions were replaced by equal volumes of water, were also analysed at suitable intervals during the experiments. The possibility that the congener may have interfered with the reaction between

TABLE 1. INHIBITION OF RAT SERUM GLUTAMIC–PYRUVIC TRANSAMINASE ACTIVITY BY SALICYLATE CONGENERS

(The results, which are expressed as mean percentage inhibitions (±s.e.m.), have been analysed by the *t*-test and values of *P* are included. The minimum acceptable level of significance has been taken as $P = 0{\cdot}05$.)

Congener	No. of observations	Concn. (mM)	% Inibition	*P*
Salicylic (2-Hydroxybenzoic)	12	1·25	6·3 ± 0·9	0·5
	12	2·5	14·0 ± 0·7	0·1
	12	5	26·4 ± 1·1	0·01
	12	10	40·0 ± 1·3	0·001
Thiosalicylic	8	sat.	46·0 ± 2·1	0·02
Benzoic	4	5	5·7 ± 1·2	0·8
3-Hydroxybenzoic	4	5	9·5 ± 1·4	0·6
4-Hydroxybenzoic	4	5	8·7 ± 1·3	0·6
2-Methoxybenzoic	4	5	7·2 ± 1·2	0·7
trans-Hexahydrosalicylic	4	5	6·2 ± 1·4	0·7
Phenol	4	5	12·5 ± 3·7	0·6
Salicylamide	4	5	7·0 ± 1·7	0·8
2-Hydroxyphenylacetic	4	5	5·2 ± 1·4	0·7
3-Methylsalicylic	8	sat.	41·7 ± 1·5	0·01
3-Ethylsalicylic	8	5	55·5 ± 0·4	0·001
3-Propylsalicylic	8	5	61·0 ± 0·6	0·001
3-*iso*Propylsalicylic	8	5	61·5 ± 0·6	0·001
3-Allylsalicylic	8	5	63·7 ± 0·9	0·001
3-Phenylsalicylic	6	5	72·0 ± 0·6	0·001
3-*cyclo*Hexylsalicylic	4	5	80·0 ± 0·9	0·001
3-Hydroxysalicylic	6	5	42·6 ± 0·8	0·01
3-Nitrosalicylic	8	sat.	33·6 ± 1·3	0·05
3-Carboxysalicylic	8	5	22·0 ± 0·9	0·01
4-Methylsalicylic	8	sat.	28·2 ± 1·2	0·05
4-Hydroxysalicylic	6	5	32·4 ± 1·0	0·05
4-Aminosalicylic	8	5	15·2 ± 0·6	0·05
5-Hydroxysalicylic	6	5	34·5 ± 1·0	0·05
5-Nitrosalicylic	8	5	51·7 ± 2·2	0·01
5-Aminosalicylic	6	sat.	43·5 ± 1·7	0·02
5-Bromosalicylic	6	5	53·3 ± 2·8	0·02
6-Hydroxysalicylic	8	5	53·6 ± 2·7	0·02
1-Hydroxy-2-naphthoic	8	5	49·1 ± 0·3	0·001
2-Hydroxy-3-naphthoic	8	5	49·9 ± 1·0	0·001
3:5-Di-*iso*propylsalicylic	7	5	76·3 ± 0·5	0·001
2:4-Dinitrophenol	6	0·5	0·6 ± 0·5	0·9

the colour reagent (2:4-dinitrophenylhydrazine) and the α-keto acids (pyruvic and α-ketoglutaric) present in the substrate, was excluded by analysing appropriate mixtures in which the serum was omitted.

RESULTS

The results are given in Table 1 and show that salicylic acid itself produced a significant inhibition of the transaminase activity at concentrations of 5 and 10 mM.

Vol. 7
1961

The hydroxyl group could be changed by replacement of the phenolic oxygen by sulphur (thiosalicylic) without loss of activity but its absence (benzoic), alteration of its position relative to the carboxyl group (3- and 4-hydroxybenzoic acids), or methylation (2-methoxybenzoic), produced inactive compounds. The corresponding hydrogenated compound (hexahydrosalicylic) was also inactive showing that the presence of a phenolic, rather than an alcoholic hydroxyl group was important for inhibitory activity. The absence of the carboxyl (phenol), the presence of an amide group (salicylamide) or the introduction of a methylene bridge between the benzene ring and the carboxyl (2-hydroxyphenylacetic) also caused a loss of activity. A general structural requirement for inhibitory activity against the transaminase in salicylate congeners therefore appeared to be a phenolic hydroxyl group in an *ortho*-position to a carboxyl group. This combination was also present in the ring-substituted salicylic acids investigated in the present work. Mono- or di-substitution in the 3-, 4-, 5- or 6-positions with a variety of groups did not remove the activity of the parent salicylic acid. Thus, the introduction of alkyl, aryl, hydroxyl, nitro and carboxyl groups in the 3-position, methyl, hydroxyl and amino groups in the 4-position, hydroxyl, nitro, amino and bromo groups in the 5-position, a hydroxyl in the 6-position, additional benzene rings in the 3:4(1-hydroxy-2-naphthoic)- and 4:5(2-hydroxy-3-naphthoic)-positions and *iso*propyl groups in the 3:5-positions, produced active compounds.

DISCUSSION

All the salicylate congeners showing inhibitory activity against rat serum glutamic–pyruvic transaminase, with the exception of thiosalicylic, contained a phenolic hydroxyl in the *ortho*-position to a carboxyl group. This combination therefore appears to be the general structural requirement for activity in this group of compounds with the reservation that a thiol group may be substituted for the hydroxyl. Substitution of the remaining positions on the benzene ring by a variety of groups did not cause a loss of activity.

Some of the salicylate congeners which inhibit the serum transaminase also uncouple oxidative phosphorylation reactions.[4] However, there is no general correlation between the two effects since the classical uncoupling reagent, 2:4-dinitrophenol had no inhibitory action in the present work. A more striking finding was that the introduction of an extra hydroxyl group in the benzene ring of salicylic acid, as in 2:5- and 2:6-dihydroxybenzoic acids, produced substances which inhibited the serum glutamic–pyruvic transaminase but which are completely devoid of uncoupling activity.[5]

Acknowledgements—We wish to thank the Medical Research Council and the Smith, Kline and French Research Institute for grants towards the cost of the work.

REFERENCES

1. M. J. H. Smith and V. Moses, *Biochem. J.* **76**, 579 (1960).
2. A. K. Huggins, M. J. H. Smith and V. Moses, *Biochem. J.* **79**, 271 (1961).
3. S. Reitman and S. Frankel, *Amer. J. Clin. Path.* **28**, 56 (1957).
4. M. J. H. Smith, *J. Pharm., Lond.* **11**, 705 (1959).
5. T. M. Brody, *J. Pharmacol.* **117**, 39 (1956); L. Packer, F. K. Austen and E. C. Knoblock, *Proc. Soc. Exp. Biol., N.Y.* **100**, 239 (1959).

Biochemical Pharmacology, 1961, Vol. 7, pp. 154–158. Pergamon Press Ltd., Printed in Great Britain.

EFFECTS OF PHYSIOLOGICAL PURINES ON THE DEVELOPMENT OF THE CHICK EMBRYO*

DAVID A. KARNOFSKY and CORINNE R. LACON

Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York

(*Received* 14 *December* 1960)

Abstract—The commonly occurring purine bases, ribonucleosides and deoxyribonucleosides were injected into the yolk sac of the 4-day-old chick embryo to determine their toxicity and teratogenic activity. The deoxyribonucleosides were most toxic, with dGuR, dAdR and dHxR having estimated LD_{50}'s of 2, 4 and 4 to 8 μmoles/egg, respectively. The deoxyribosyl derivatives caused marked disturbances in development of the 4-day embryo, with stunting of growth, edema, facial defects, absent bones and shortened extremities. dGuR was most active in producing weight inhibition and severe developmental disturbances; these effects were seen at 1 μmole/egg for dGuR, in contrast to 16 μmoles/egg for dHxR, and at an excess of 8 μmoles/egg for dAdR.

Vol. 7
1961

A NUMBER of antimetabolites which interfere with purine and pyrimidine synthesis[1] inhibit the normal growth and development of the chick embryo. Thus, the folic acid antagonists,[2] the glutamine antagonists[3] and the fluorinated pyrimidines[4] are toxic to the chick embryo, and at sublethal doses they induce severe developmental disturbances. Certain physiological purines can prevent the effects of specific antimetabolites by presumably restoring the substance whose production is blocked by the antimetabolite.[5]

In determining the amounts of physiological purines tolerated by the chick embryo during these protection studies, it was noted that the deoxyribosyl derivatives were more toxic than the purine bases or their ribosyl derivatives, and the deoxyribosides also produced developmental disturbances in the surviving embryos.

MATERIALS AND METHODS

Fertile white Leghorn eggs, obtained from a commercial source, were incubated at 38 °C. In most experiments, the drug was injected into the yolk sac through a small hole, produced in the blunt end of the egg, on the fourth day of incubation. The eggs were candled daily, and the dead ones recorded. Those embryos dying between the fifth and tenth days of incubation were discarded, but from the eleventh to eighteenth days the dead embryos were inspected and gross abnormalities recorded. Embryos surviving to the eighteenth day of incubation were sacrificed. These embryos were weighed, inspected for gross abnormalities, and in many instances they were cleared and the skeleton stained with alizarin.[6]

The chemicals were made up in a suspension of 0·5 per cent carboxymethyl cellulose in saline. The volume injected into each egg did not exceed 0·2 ml.

In a few instances drugs were given at 2, 8 or 12 days of incubation. The chemicals given, and the abbreviations used in this paper are: guanine (Gu), guanosine (GuR),

* This research was supported, in part, by grants from the American Cancer Society, Inc (T-40), the Albert and Mary Lasker Foundation, and grants from the National Institutes of Health.

2′-deoxyguanosine (dGuR),* adenine (Ad), adenosine(AdR), 2′-deoxyadenosine (dAdR),* hypoxanthine (Hx), inosine (HxR) and 2′-deoxyinosine (dHxR).*

RESULTS

The toxicity of the physiological purines following yolk sac injection at 4 days of incubation is summarized in Table 1. The number of eggs used, the dosage range and an approximate LD_{50}, estimated from the number of embryos surviving at 10 days following injection, are recorded for each drug. An LD_{50} was estimated from the results of several separate experiments, using the dose ranges shown in Table 1. Toxicity data are variable in separate experiments so that a precise LD_{50} is not useful. The incidence of developmental abnormalities is calculated from the examination of embryos from 11–18 days of incubation, and the surviving embryos sacrificed at 18 days. The most toxic purines in the chick embryo are the 2′-deoxyribosyl derivatives of guanine, adenine and hypoxanthine, in that order, in comparison to the less toxic purine bases and ribosyl derivatives. The deoxyribosyl derivatives also produced developmental abnormalities, and again dGuR proved to be most active.

TABLE 1. TOXICITY OF THE PHYSIOLOGICAL PURINES INJECTED INTO THE YOLK SAC OF THE 4-DAY CHICK EMBRYO

(Survivors sacrificed at 18 days of incubation.)

Compound	Dose range (mg/egg)	No. eggs injected 4 days	No. embryos alive		Abnormal embryos 11–18 days		Approximate LD_{50}	
			10 days	18 days	No.	%	mg/egg	μmoles/egg
Ad	2–8	55	29	23	2	7	4	30
AdR	2–8	55	40	29	0	0	2–4	8–16
dAdR	0·5–4	187	108	85	35	32	1	4
Gu	2–8	65	45	39	0	0	8	53
GuR	2–12	65	49	45	6	12	8	28
dGuR	0·1–4	277	193	153	133	69	0·5	2
Hx	2–8	45	21	18	0	0	2–4	15–30
HxR	2–8	35	22	17	2	9	4–8	15–30
dHxR	0·5–4	127	80	59	29	36	1–2	4–8

The effects of the deoxyribosyl purines were compared simultaneously in one experiment (Table 2). The differences noted were confirmed in a number of other experiments (Table 1). dGuR was highly teratogenic at 0·25 mg/egg, and larger doses produced 100 per cent of abnormal embryos. dAdR was about half as toxic dGuR, but even above the LD_{50} dAdR was weakly teratogenic in embryos surviving to 18 days of incubation. dHxR was least toxic, -and definite teratogenic effects occurred at 4·0 mg/egg. Accordingly, there appeared to be some dissociation between the toxicity and the weight inhibition and teratogenic effects of the deoxyribosides. dAdR at LD_{50} doses at 4 days caused less severe weight inhibition and inconsistent developmental effects in the embryos surviving beyond 10 days, as compared to equivalent toxic doses of dGuR.

The teratogenic effects of dGuR were analyzed in data pooled from several separate experiments (Table 3). Weight inhibition and developmental abnormalities were severe, and were particularly conspicuous in the embryos surviving and sacrificed on the eighteenth day of incubation (Fig. 1). The embryos grossly were stunted and edematous.

* The deoxyribonucleosides were obtained from the California Corporation for Biochemical Research.

TABLE 2. COMPARATIVE TOXICITY AND TERATOGENIC EFFECTS OF THE DEOXYRIBOSYL DERIVATIVES OF THE PHYSIOLOGICAL PURINES
(Injected into the yolk sac at 4 days of incubation; survivors sacrificed at 18 days.)

	2′-Deoxy-guanosine			2′-Deoxy-adenosine			2′-Deoxy-inosine		
Dose (mg/egg)	0·25	0·5	1·0	0·5	1·0	2·0	1·0	2·0	4·0
No. injected, 4 days	20	20	20	20	20	20	20	20	20
No. sacrificed, 18 days	16	9	5	16	7	4	14	5	8
No. of abnormal embryos	12	9	5	0	2	2	2	1	8
Abnormalities:									
Facial coloboma and cleft palate	7	9	5		1				2
Cleft palate	1						1		
Short lower beak	7	8	5		2	2		1	7
Micromelia	12	9	5						
Missing toes	9	9	5						3
Short toes	3								2
Webbed toes	4						1		2
Corneal cysts	1	6	5						
Eyelid defect		1							
Exteriorized viscera			1						
Edema, localized	2	1	2						1
Edema, generalized	3	7	4		1				
Feather inhibition	2	2	3						
Average emb. (wt., g)	12·9	10·4	8·5	15·6	14·1	11·5	15·7	14·5	13·0
18 days s.d.	±1·1	±2·3	±1·7	±2·6	±2·0	±1·1	±1·5	±2·6	±1·6

TABLE 3. TOXICITY AND TERATOGENIC EFFECTS OF 2-DEOXYGUANOSINE
(Injected into the yolk sac at 4 days of incubation; survivors sacrificed at 18 days.)

Dose (mg/egg)	0·1	0·25	0·50	1·0
No. injected, 4 days	18	107	85	59
No. sacrificed, 18 days	15	79	45	14
No. of abnormal embryos	1	49	44	14
Abnormalities:				
Facial coloboma and cleft palate	1	23	41	14
Cleft palate		6		
Short lower beak		24	40	13
Micromelia		42	43	14
Missing toes		37	42	14
Short toes		20	7	1
Webbed toes		10	4	
Corneal cysts		1	28	11
Eyelid defect		3	4	2
Exteriorized viscera				1
Edema, localized		5	3	5
Edema, generalized		7	18	8
Feather inhibition		4	2	3
Average embryo weight; s.d.	16·0 ±2·2	13·7 ±2·7	10·4 ±2·2	9·7 ±2·3

Many had corneal cysts, with occasional eyelid defects. Feather growth was often inhibited, and the extremities were grossly deformed, with shortened legs and wings and missing toes. The heads were severely distorted with short lower beak and cleft palates.

The gross disturbances corresponded with the bony changes noted in the cleared embryos. Some of the embryos treated at 0·25 mg/egg showed facial coloboma, cleft palate, shortened lower beaks and fusion of the cervical vertebrae. Many had fusion of the humerous and radius and of the femur and tibia. The metatarsals showed fusion and shortening, and the second and fourth toes were often missing.

Vol. 1961

Vol. 7
1961

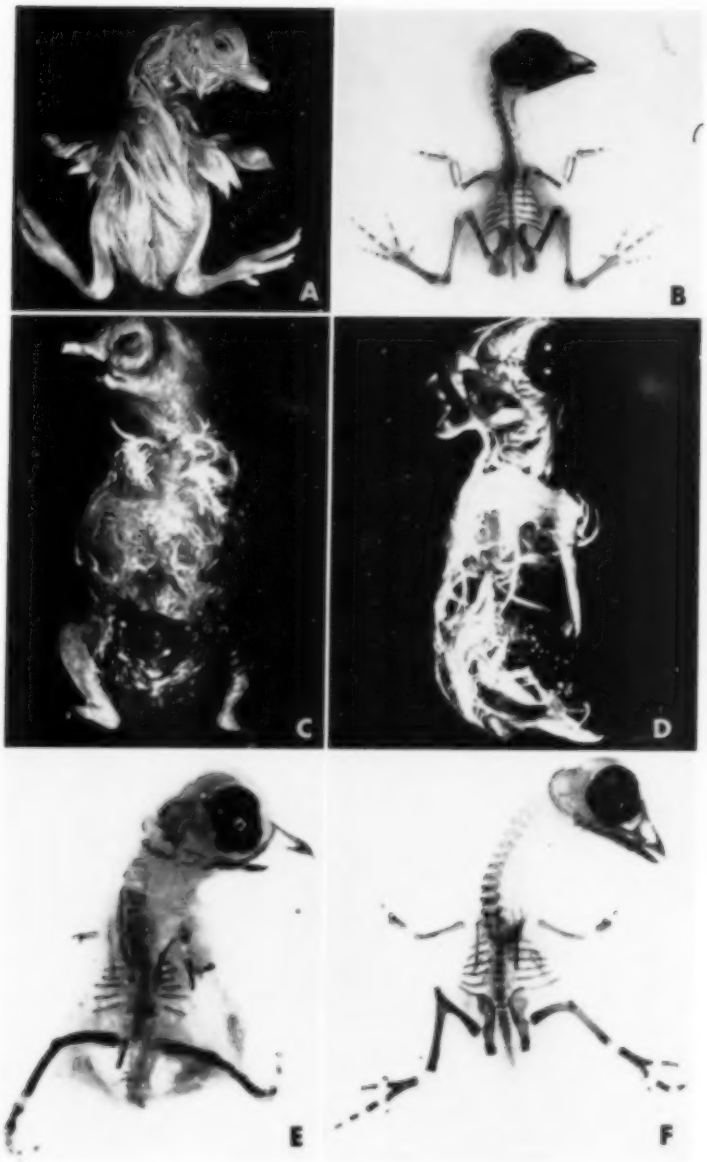


FIG. 1. (Photographs not to scale).

(caption on reverse)

facing page 156

Caption Fig. 1.

A. Normal 18-day chick embryo.

B. Alizarin-stained skeleton of normal 18-day chick embryo.

C. Eighteen-day chick embryo, treated at 4 days with 0·5 mg of 2′-deoxyguanosine injected into the yolk sac. The embryo is moderately stunted and markedly edematous. There is a facial coloboma, a cleft palate and a short lower beak. Feather development is abnormal. There is marked shortening of the metatarsals and metacarpals, with only a single toe on each leg.

D. Eighteen-day chick embryo treated at 4 days with 0·5 mg of 2′-deoxyguanosine. The egg also received 4 mg of guanosine which does not affect the action of dGuR. The embryo is stunted. There are large corneal cysts. A facial coloboma and cleft palate are present. The long bones are shortened. There is incomplete closure of the lower abdominal wall.

E. Alizarin-stained, cleared skeleton of an 18-day chick embryo, treated at 4 days with 0·5 mg of 2′-deoxyguanosine injected into the yolk sac. The embryo is severely stunted and edematous. There is absence of calcified facial bones except the premaxilla (incisive); there is a short lower beak, the vertebrae are fused in the cervical and thoracic region, and poorly formed, the ribs are short and of irregular length, the femur and tibia are fused and the fibula missing; wings are poorly formed and the metacarpals and phalanges are incomplete.

F. Alizarin-stained, cleared skeleton of 18-day chick embryo treated at 4 days with 0·25 mg of 2′-deoxyguanosine injected into the yolk sac. The head is normal. The vertebrae appear normal. The humerus–radius and femur–tibia are fused, the ulna and fibula are missing. The terminal digits are poorly formed and toes 1 and 4 are shortened.

At 0·5 mg/egg the severe changes were more consistent, with face and rib defects, fusion of long bones, and absence of the ulnae. In some embryos the metacarpals and phalanges were missing. Embryos surviving 1·0 mg/egg until the eighteenth day were affected most severely. Facial bones were almost entirely absent, vertebrae were fused in a random manner, ribs were short and uneven, the long bones were fused, and metacarpals, metatarsals and phalanges missing. The ilium was missing in some cases, and the ischium and pubis were absent in all animals examined at this dose.

The LD_{50} of dGuR injected into the yolk sac at 2 days of incubation was approximately 0·05–0·10 mg/egg. Teratogenic effects were observed in embryos dying within 10 days after injection, but those surviving to 18 days were grossly normal. The toxicity and teratogenic action of dGuR thus appear to be increased in the 2-day as compared to the 4-day embryo, in that those affected did not survive to 18 days. At from 8 to 12 days of incubation, the toxic effects of dGuR and dAdR injected into the yolk sac were markedly reduced, and doses up to 10 mg/egg were without effect.

DISCUSSION

Of the physiological purines studied, the deoxynucleosides, particularly dGuR, were uniquely active in producing growth inhibition and developmental abnormalities. Distinct differences between toxicity and teratogenic activity were observed when the embryos were treated at 4 days (Table 4).

TABLE 4.

	dGuR	dAdR (μmoles/egg)	dHxR
Approximate LD_{50}	2	4	4–8
Effective teratogenic dose	1	8	16

While abnormal purine deoxynucleosides have not been available for study thus far, the toxicity of the physiological deoxyribonucleosides is consistent with observations on the enhanced activity of certain abnormal pyrimidine deoxyribonucleosides. When injected into the yolk sac of the 4-day embryo, 5-fluorodeoxyuridine may be lethal or teratogenic at 0·05 μg/egg, whereas comparable effects occur with 3·0 μg of 5-fluorouridine per egg and 200 μg of 5-fluorouracil per egg.[4] 5-Bromo- and 5-iodo-deoxyuridine cause growth inhibition when injected into the yolk sac of the 4-day embryo at doses of 50 and 75 μg/egg, respectively, whereas 5-bromo- and 5-iodo-uracil and their ribosyl derivatives are non-toxic up to 5000 μg/egg.[7] These data suggest that the embryo, *in vivo*, at 4 days may be unusually susceptible to deoxyribonucleosides, possibly because it cannot readily catabolize these compounds. Furthermore, the lower toxicity of the purine and pyrimidine bases and nucleosides suggest that the 4-day embryo cannot readily convert them to the more toxic deoxyribonucleosides. If this generalization can be supported by direct biochemical evidence, the 4-day chick embryo represents a favorable environment in which to study the inherent biological activity of preformed deoxyribonucleosides. Reichard, however, found that soluble enzymes extracted from 5-day old chick embryos reduced ribonucleotides of uracil, cytosine[8] and guanine[9] to their corresponding deoxyribonucleotides. This is not necessarily consistent with our *in ovo* observation that guanosine is neither highly toxic nor teratogenic, and it should be noted that 5′-guanylic acid is also comparatively nontoxic (approximate LD_{50}, 5 mg/egg) and non-teratogenic.

Inhibitory effects of dAdR on nucleic acid metabolism *in vitro* have been reported. Klenow and Langer[10] found that dAdR inhibited the incorporation of ^{14}C-formate into the DNA-thymine of Ehrlich cells *in vitro*; in contrast, dAdR had little effect on Yoshida ascites cells. The amount of acid-soluble thymine compounds was increased in the Ehrlich ascites cells treated with dAdR, indicating that dAdR did not interfere with thymine formation but with the synthesis of DNA.[11]

The effect of dAdR was reversed by dGuR, but not dHxR,[12] suggesting that dAdR inhibited the formation of dGuR from a closely related precursor. Prusoff[13] also found that dAdR blocked the utilization of ^{14}C-formate for the biosynthesis of DNA-thymine. In the most recent report, Maley and Maley[14] demonstrated that by incubating 4-day chick embryo suspensions for 2 hr *in vitro* with dAdR, cytidine-2-^{14}C and uridine-2-^{14}C incorporation was blocked, and the incorporation of thymidine-^{3}H into DNA was partially inhibited. While dGuR was less active than dAdR, it also inhibited the incorporation of precursors into DNA.

Our *in vivo* results indicate that dGuR is more active than dAdR and dHxR, particularly in regard to teratogenic activity. Our results thus differ quantitatively from those obtained with the chick embryo,[14] since dAdR is more active than dGuR *in vitro*, and the reverse of the *in vitro* results on Ehrlich ascites cells, as reported by Klenow and Langer[10], and Prusoff,[13] since they found dGuR ineffective.

dGuR causes profound developmental disturbances in the 4-day chick embryo consisting of growth inhibition, edema, beak defects and skeletal disturbances, particularly of the extremities. Similar effects have been seen in 4-day embryos treated with the glutamine antagonist, O-diazoacetyl-l-serine (azaserine)[3] and the fluorinated pyrimidines.[4] The teratogenic effects of azaserine can be prevented by adenine and the purine nucleosides,[5] although guanine is conspicuously ineffective, and thymidine will protect against the teratogenic and toxic effects of 5-fluorodeoxyuridine.[7] Azaserine, 5-fluorouracil and dGuR cause their most striking effects in embryos treated at 4 days of incubation; the high level of teratogenic activity at 4 days is in contrast to drugs which exhibit maximal effects at 8 days; these include cortisone,[6] insulin,[15] nicotanimide antagonists and thallium.[16] These data suggest the chick embryo at 4 days is unusually susceptible to drugs which interfere with nucleic acid metabolism.

REFERENCES

1. I. H. KRAKOFF, *Amer. J. Med.* **28**, 735 (1960).
2. D. A. KARNOFSKY, P. A. PATTERSON and L. P. RIDGWAY, *Proc. Soc. Exp. Biol., N.Y.* **71**, 447 (1949).
3. C. P. DAGG and D. A. KARNOFSKY, *J. Exp. Zool.* **130**, 555 (1955).
4. D. A. KARNOFSKY, M. L. MURPHY and C. LACON (Abstr.), *Proc. Amer. Ass. Cancer Res.* **2**, 312 (1958).
5. C. P. DAGG and D. A. KARNOFSKY (Abstr.), *Fed. Proc.* **15**, 238 (1956).
6. M. H. MOSCONA and D. A. KARNOFSKY, *Endocrinology* **66**, 533 (1960).
7. D. A. KARNOFSKY and C. R. LACON, Unpublished data.
8. P. REICHARD, *Biochim. Biophys. Acta* **27**, 434 (1958).
9. P. REICHARD, *Biochim. Biophys. Acta* **41**, 368 (1960).
10. H. KLENOW and L. LANGER, *Acta Chem. Scand.* **12**, 1355 (1958).
11. H. KLENOW, *Biochim. Biophys. Acta* **35**, 412 (1959).
12. L. LANGER and H. KLENOW, *Biochim. Biophys. Acta* **37**, 33 (1960).
13. W. H. PRUSOFF, *Biochem. Pharmacol.* **2**, 221 (1959).
14. B. F. MALEY and V. MALEY, *J. Biol. Chem.* **235**, 2964 (1960).
15. W. LANDAUER, *J. Exp. Zool.* **105**, 145 (1947).
16. D. A. KARNOFSKY, *Stanford Med. Bull.* **13**, 247 (1955).

Vol. 7
1961

ANNOUNCEMENT

The Fourth Annual Meeting of the American Association of Poison Control Centers

THIS meeting will take place on October 3, 1961, during the meeting of the American Academy of Pediatrics. It will be held at:

Wabash Parlor of the Palmer House,
Chicago, Illinois, U.S.A.

Papers are solicited for presentation at this meeting. There is no particular limitation as to the type of paper to be presented, except that it should deal with either some general or special aspect of toxicology, or other aspects of poisoning or poison control.

The abstract for each paper should be sent to HARRY C. SHIRKEY, M.D., The Children's Hospital, Birmingham, Alabama, on or before August 1.

Papers to be published in future issues

U. Bardi, Giulia Boretti and A. Di Marco: Inhibition of oxidative phosphorylation by aminosidine and other antibiotics in rat liver mitochondria.

D. L. Smith and E. D. Harris, Jr.: The reduction of triphenyltetrazolium by hypertrophied tissues.

A. Ben and R. Singer-Altbeker: The influence of thiamine and related compounds on the iris of the frog.

R. G. Wiegand and J. E. Perry: Effect of L-DOPA and N-methyl-2-propynylamine on DOPA, dopamine, norepinephrine, epinephrine and serotonin levels in mouse brain

J. F. Henderson and I. G. Junga: Effects of purine antagonists on the metabolism of other purine antagonists.

S. H. Ngai, S. Ginsburg and R. L. Katz: Action of thiamine and its analogues on neuromuscular transmission in the cat.

I. Enander, A. Sundwall and Bo Sörbo: Metabolic studies on N-methylpyridinium-2-aldoxime—I. The conversion to thiocyanate.

I. Enander, A. Sundwall and Bo Sörbo: Metabolic studies on N-methylpyridinium-2-aldoxime—II. The conversion to N-methylpyridinium-2-nitrile.

W. D. Wosilait: The reduction of cytochrome *c* by reduced vitamin K_1.

A. M. Rothschild, I. Vugman and M. Rocha e Silva: Metabolic studies on the release of histamine by compound 48/80 in the rat diaphragm.

L. Levy and G. E. Cronheim: Blood levels and clearing activity of a synthetic heparinoid.

J. A. Fouts, R. L. Dixon and R. W. Shultice: The metabolism of drugs by regenerating liver.

C. Van der Meer and P. W. Valkenburg: The pharmacology of KCN as a prophylactic against radiation.

T. Zsolnai: Versuche zur Entdeckung neuer Fungistatika—III. 8-Hydroxy-chinolin-Derivate, Nitroso-Verbindungen und Oxyme.

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 161–164. Pergamon Press Ltd., Printed in Great Britain.

THE REDUCTION OF TRIPHENYLTETRAZOLIUM BY HYPERTROPHIED TISSUES*

DONN L. SMITH and EDWARD D. HARRIS, JR.†

Department of Pharmacology, University of Colorado School of Medicine, Denver, Colorado

(*Received* 17 *January* 1961)

Abstract—Hypertrophy of the myocardium, adrenal gland and thyroid gland was induced in adult male and female albino rats. Tetrazolium reductase levels of the hypertrophied tissues were determined by means of the triphenyltetrazolium technique. Control and experimental organ weights were measured to quantitate increases in tissue mass. No significant change occurred in the endogenous levels of dehydrogenase in cardiac or adrenal gland tissue, when work hypertrophy was induced in those tissues. Increased gland weight or increased gland work in the case of the thyroid gland was found to be associated with increases in tetrazolium reductase levels which were significantly elevated above control levels. In cardiac tissue slices from bilaterally thryroidectomized animals significant reductions in endogenous dehydrogenase levels were observed. The implications of these observations are discussed.

TETRAZOLIUM salts have been employed in recent years as indicators of changes in tissue metabolism under a variety of experimental conditions.[1–3] Although the specific metabolic implications of the reduction of tetrazolium by tissues are partially speculative, the reaction serves as a useful method for the determination of metabolic alterations in tissue slices.[4] The current status of the tetrazolium technique has been well reviewed by Pearse.[5] Triphenyltetrazolium chloride is desirable for use in quantitative studies because of its low toxicity for tissues, the monochromatic character of the formazen resulting from the dye reduction, and a high rate of diffusion into tissues.

This investigation was designed to determine if hypertrophy or hyperplasia of various tissues could be related to metabolic changes within the tissues, as indicated by quantitative alterations in the reduction of triphenyltetrazolium chloride by tissues.

METHODS

Hypertrophy was induced in thyroid, adrenal and cardiac tissues of male and female adult albino rats of the Denver strain by the following methods.

(a) *Thyroid.* Thiouracil was administered in the drinking water as a 0·1 per cent solution for 10 days. A second series of animals was subjected to surgical hemithyroidectomy of the right lobe.

(b) *Adrenal.* A group of animals was subjected to unilateral adrenalectomy, the right adrenal gland being removed.

(c) *Heart.* Animals were injected subcutaneously for 14 days with synthetic l-thyroxin (as synthroid Na) in saline in a dose of 75 μg/rat per day. l-Tri-iodothyronine

* Supported in part by a grant (H-2664) from the National Heart Institute.

† Present address: Harvard Medical School.

was administered subcutaneously for 14 days to a second group of rats in a dose of 50–75 μg/rat per day. A third series of animals was subjected to bilateral thyroidectomy.

Endogenous dehydrogenase activity of the tissues was measured by the quantitative tetrazolium reduction technique,[6] as modified for use with tissue slices.[7] Triphenyltetrazolium chloride was employed as the indicator in a modified Krebs solution,[4] at a pH of 7·4. This pH was maintained in all determinations because of the possibility of non-specific reduction of tetrazolium at higher pH values, as shown by Seligman.[8] Aerobic incubation of the tissues was carried out for 2 hr at 37 °C with shaking at 60 cycles/min. The amount of dye reduced by the tissue after extraction with acetone, was quantitated by colorimetry at 470 mμ. All values thus obtained were converted to μg of dye reduced per mg of air-dried tissue. Control tissues were inserted in each group of experimental determinations.

All animals were killed by a sharp blow on the head and the appropriate tissue was rapidly removed, sliced and incubated. The experimental periods were as follows: thiouracil administration, 10 days; unilateral thyroidectomy, 7 days; l-thyroxine and l-tri-iodothyronine administration, 14 days; bilateral thyroidectomy, 7 days; unilateral adrenalectomy, 7 days. Heart weights represent the total wet weight of the right and left ventricles and the intraventricular septum. Adrenal and thyroid glands were air-dried after acetone extraction and weighed.

Vol. 7
1961

TABLE 1. ORGAN WEIGHTS AND TISSUE DEHYDROGENASE ENZYME ACTIVITY

Organs and tissues	No. animals	Mean weight of organs in mg. *	s.e.	No. of animals	μg of dye reduced/mg dry tissue mean	s.e.
Thyroid						
Control	16	1·57	± 0·08	16	15·3	±0·63
Unilateral thyroidectomy	14	1·70†	± 0·05	14	19·4	±0·74‡
Thiouracil 0·1%	19	5·00	± 0·30‡	19	35·4	±0·94‡
Adrenal						
Control	35	2·33	± 0·09	34	25·0	±0·63
Unilateral adrenalectomy	10	3·93	± 0·25‡	10	24·1	±0·64
Control	59	322·90	± 3·43	18	21·1	±0·55
l-Thyroxine	32	417·00	± 6·80‡	12	21·8	±0·94
l-Tri-iodothyronine	9	478·00	±19·80‡	9	20·0	±0·72
Bilateral thyroidectomy	24	296·00	± 6·30	20	16·5	±0·56‡

* Thyroid and adrenal weights, air-dried; heart weights, wet tissue/100 g body weight.

† Weight of remaining lobe × 2.

‡ Difference is statistically significant, $P < 0·0001$, when compared to control values.

RESULTS AND DISCUSSION

The results of this study are tabulated in Table 1. Although significant increases in both heart and adrenal gland weights were observed, no change occurred in the endogenous levels of the dehydrogenase of the hypertrophied tissues. The enzyme levels observed in the control adrenal glands are in close agreement with those

reported by other workers.[9] These data suggest that the increases in tissue mass which were demonstrated were not accompanied by an increase in the functional metabolic processes related to reductase activity within the cells of adrenal gland or cardiac tissue.

In thyroid glands rendered hyperplastic and hypertrophic by thiouracil administration a significant increase in reductase levels was demonstrated in association with the increase in tissue mass. The lobes of the thyroid gland remaining after hemi-thyroidectomy showed an elevated endogenous dehydrogenase level in the absence of increased tissue weight. Although there was no perceptible increase in the reductase level in cardiac tissue from animals exposed to relatively large amounts of l-thyroxine or l-tri-iodothyronine, the relative lack of endogenous thyroxine in the bilaterally thyroidectomized animals was associated with a reduced tissue level of dehydrogenase in the myocardium.

The question of primary interest raised by the results of this study concerns the lack of response of the reductase level to hypertrophy of the cardiac muscle and the adrenal gland tissue, as contrasted to the large elevation of tetrazolium reduction observed in the enlarged thyroid and in thyroid tissue after hemi-thyroidectomy. It is possible that the metabolic response to increased tissue mass in the heart and the adrenal gland involved enzyme systems which are not DPN- or TPN-dependent reductases. Cascarano and Zweifach[10] have shown that tetrazolium reduction in tissues slices is related to substrate formation by DPN- and TPN-dependent dehydrogenases. It would follow, then, that metabolic changes occurring in hypertrophied or hyperplastic cells and mediated by enzymes other than such dehydrogenases, would not be reflected in changes in the levels of tetrazolium reductase. This fact has been demonstrated in the case of the adrenal glands by histochemical methods.[11] Increases in endogenous dehydrogenase were recorded in both enlarged thyroid glands and in non-enlarged thyroid gland lobes remaining 7 days following hemi-thyroidectomy. Therefore, hypertrophy alone is not a primary casual factor in the elevation of reductase levels in thyroid tissue. Under the conditions which prevailed in these experiments it is likely that increased functional activity in thyroid cells, as opposed to similar activity in cardiac or adrenal cells, is the initiating factor for the observed effect on reductase activity which is specific for thyroid tissue. It is apparent that under these circumstances stimulation of thyroid tissue by thyrotropin may be the operative mechanism in the elevation of certain endogenous dehydrogenases. It has been shown that chronic administration of l-thyroxine with a concomitant reduction of thyrotropic stimulation results in decreased reductase levels in thyroid tissues.[12] It is possible that stimulation of thyroid gland cells by thyrotropin causes an increase in oxidative potential within the cells which is reflected by equivalent increases in DPN- or TPN-dependent dehydrogenases.

The significant decrease in reductase levels which occurred in cardiac tissue of thyroidectomized animals indicates simply that physiological amounts of thyroid hormone are required for maintenance of normal reductase levels in the myocardium. Relatively large amounts of circulating amounts of thyroid hormones did not increase dehydrogenase levels in cardiac tissue. An explanation for this finding may lie in the reports of Barker[13] and Laursen[14] which show that endogenous dehydrogenase activity, as measured by tetrazolium reduction, represents only from 3 to 5 per cent of the total metabolic activity of tissues expressed as oxygen consumption.

The data herein presented support the conclusion that alterations in cellular reductase levels in thyroid tissue are associated with increases in specific cellular function. Hypertrophy *per se* does not initiate changes in the activity of dehydrogenases dependent upon DPN or TPN in cardiac or adrenal gland tissue. The relationship between thyrotropic stimulation of the thyroid gland and endogenous tetrazolium reductase activity in thyroid cells represents an interesting problem for future study.

REFERENCES

1. M. M. BLACK and J. S. KLEINER, *Science* **110**, 660 (1949).
2. M. M. BLACK, S. R. OPLER and F. D. SPEER, *Amer. J. Path.* **26**, 1097 (1950).
3. O. H. IVERSON, *Acta. Path. Microbiol. Scand.* **47**, 216 (1959).
4. E. KIVY-ROSENBERG, J. CASCARANO and B. W. ZWEIFACH, *Trans. N.Y. Acad. Sci.* **17**, 402 (1955).
5. A. G. E. PEARSE, *J. Clin. Path.* **11**, 520 (1958).
6. E. KUN and L. G. ABOOD, *Science* **109**, 144 (1949).
7. M. M. BLACK, B. W. ZWEIFACH and F. D. SPEER, *Amer. J. Clin. Path.* **23**, 332 (1952).
8. A. M. SELIGMAN and A. M. RUTENBERG, *Science* **113**, 317 (1951).
9. J. CASCARANO and B. W. ZWEIFACH, *J. Histochem. Cytochem.* **3**, 369 (1955).
10. J. CASCARANO and B. W. ZWEIFACH, *J. Biophys. Biochem.* **5**, 309 (1959).
11. B. W. ZWEIFACH, M. M. BLACK and E. SHORR, *Proc. Soc. Exp. Biol., N.Y.* **76**, 446 (1951.
12. M. E. BIERWAGEN and D. L. SMITH, *Proc. Soc. Exp. Biol., N.Y.* **100**, 108 (1959).
13. S. B. BARKER, *Endocrinology* **57**, 414 (1955).
14. T. J. S. LAURSEN and R. S. LAURSEN, *Amer. J. Clin. Path.* **30**, 237 (1958).

Vol.
196

Biochemical Pharmacology, 1961, Vol. 7, pp. 165–168. Pergamon Press Ltd., Printed in Great Britain.

INHIBITION OF OXIDATIVE PHOSPHORYLATION BY AMINOSIDINE AND OTHER ANTIBIOTICS, IN RAT LIVER MITOCHONDRIA

U. Bardi, Giulia Boretti and A. Di Marco

Laboratori Ricerche Farmitalia, Milano

(*Received* 6 *February* 1961)

Abstract—Several antibiotics, belonging to the class of basic polysaccharides, such as streptomycin, kanamycin, neomycin, and aminosidine, inhibit the phosphorylation coupled to ketoglutarate oxidation in rat liver mitochondria. The effect of aminosidine has been compared to that of its N-methaneosulphonate derivatives and of the products obtained by methanolysis. Results show that the uncoupling activity depends both on the presence of free amino groups and on the moelcular complexity of the antibiotic. The mechanism of the uncoupling effect of aminosidine is different from that of dinitrophenol.

Vol. 7
1961

The present paper* deals with the study of the action of the antibiotics streptomycin, kanamycin, neomycin, aminosidine,[1] and trehalosamine[2] on the phosphorylation coupled to ketoglutarate oxidation in rat liver mitochondria. The antibiotics examined have in common the structure of basic oligosaccharides.

The relation between chemical structure and uncoupling effect on the oxidative phosphorylation of mitochondria has been studied particularly with regard to aminosidine. This antibiotic is a pentabasic oligosaccharide having the formula $C_{23}H_{45}N_5O_{14}$. It is split, by acid hydrolysis, into a glucoside ($C_{12}H_{25}N_3O_7$) and an aminodisaccharide (probably $C_{11}H_{22}N_2O_8$). Two substances, a crystalline glucoside ($C_{12}H_{25}N_3O_7 \cdot 3HCl$) and the methylglucoside of an aminodisaccharide [$C_{11}H_{21}N_2O_7(OCH_3)$, $2HCl$][3] have been obtained by methanolysis. Analogous products have been obtained from paromomycin[4] and hydroxymicin.[5] The treatment of the antibiotic with sodium bisulphate and formaldehyde gives rise to methanesulphonates, analogously to what occurs with kanamycin.[6]

MATERIAL AND METHODS

We used the following products: adenosinetriphosphate (sodium salt) (Boehringer), hexokinase Sigma type II, cytochrome *c* (Boehringer), ketoglutarate (Biosintex), β-hydroxybutyric acid (Fluka), streptomycin sulphate (Farmitalia), kanamycin sulphate (Merck), neomycin sulphate (Farmitalia.) Aminosidine sulphate, trehalosamine sulphate, as well as the products obtained by methanolysis of the latter and the N-methanesulphonates of aminosidine, have been prepared in our laboratory.

Rat liver mitochondria were prepared according to Schneider.[7] The oxidative phosphorylation activity was determined in suspensions of mitochondria in 3 ml of medium containing sucrose 250 μM, EDTA 2μM, K malonate 30 μM, K ketoglutarate 30 μM, KH_2PO_4 40 μM, cytochrome *c* 0·06 μM, ATP 6 μM, KF 120 μM, $MgSO_4$

* The following abbreviations are used: EDTA = ethylenediaminotetra-acetic acid; ADP = adenonine diphosphate; ATP = adenosine triphosphate; Pi = inorganic phosphorus; DNP = dinitrophenol.

50 μM, glucose 40 μM, hexokinase 0·9 mg. The mixture was incubated in Warburg flasks for 20 min at 30 °C. Oxygen uptake was measured by Warburg manometric technique. Inorganic phosphate was determined, according to Marsh,[8] in the supernatant of the suspension deproteinized by 0·5 ml 20% TCA, at zero time and at the end of incubation. Experiments in phosphate or phosphate acceptor deficient systems were performed using glutamate as substrate, according to Lardy and Wellman's method.[9] ATPase activity of mitochondria was determined according to Myers and Slater in Tris buffer at pH 7·4.[10] Hexokinase activity was determined by Colowick and Kalkar's method.[11] Ion-complexing activity of aminosidine was determined by Albert's method.[12]

RESULTS

Among the antibiotics tested, streptomycin, kanamycin, aminosidine and neomycin, at concentrations ranging from 10 to 50 μg/ml, inhibit the phosphorylation coupled to ketoglutarate oxidation in rat liver mitochondria and show about the same degree of activity (Table 1). Trehalosamine, which is a monobasic disaccharide, is inactive.

Table 1. Influence of some antibiotics on P : O of rat liver mitochondria

No additions		Aminosidine		Streptomycin		Neomycin		Kanamycin		Trehalosamine	
O_2 μatoms	P:O	O_2 μatoms	P:O	O_2 μatoms	P:O	O_2 μatoms	P:O	O_2 μatoms	P:O	O_2 μatoms	P:O
3·65	3·13	3·82	1·84	4·39	1·85	3·65	1·52	3·40	1·34	5·73	2·31
3·98	2·66	3·65	1·69								
3·46	2·32	3·38	1·53								
5·10	2·56	5·31	1·63								

Substrate: ketoglutarate. Concentrations of added antibiotics = 50 μg/ml.

Table 2. Influence of N-methanesulphonate derivatives of aminosidine on P : O of rat liver mitochondria

	No additions	Aminosidine 50 μg/ml	MS_4 50 μg/ml	MS_5 50 μg/ml
P:O	3·08	1·62	3·12	2·86

Substrate: ketoglutarate.
MS_4 = aminosidine-di-N-methanesulphonate.
MS_5 = aminosidine-tetra-N-methanesulphonate.

The action of aminosidine has been compared to that of its methanesulphonate derivatives and to that of the products obtained by methanolysis and we have observed that none of the derivatives of aminosidine interferes with the processes of oxidative phosphorylation (Tables 2 and 3). Aminosidine inhibits oxidative phosphorylation also with β-hydroxybutyrate and glutamate as substrates. Its action is not due to an interference with the mechanism of transfer of the terminal phosphate of ATP on glucose; indeed it does not inhibit hexokinase at the concentrations used in the tests for oxidative phosphorylation, moreover, in this case it would cause an inhibition of O_2 consumption due to a depletion of ADP. Aminosidine neither stimulates the QO_2 of mitochondria in Pi or ADP deficiency (Table 4), nor influences QO_2 stimulation induced by dinitrophenol (DNP) under these conditions. Aminosidine, contrary to

Vol. 7
1961

TABLE 3. INFLUENCE OF AMINOSIDINE AND OF THE SUBSTANCES OBTAINED BY METHANOLYSIS OF AMINOSIDINE ON P:O OF RAT LIVER MITOCHONDRIA

	No additions	Aminosidine 50 μg/ml	α	β	α + β
P:O	2·35	1·21	2·37	2·20	
	2·67	1·40			
	2·78	1·58			2·54

Substrate: ketoglutarate.
α and β are, respectively, $C_{12}H_{25}N_3O_7 \cdot 3HCl$ and $C_{11}H_{21}N_3O_7(OCH_3) \cdot 2HCl$ obtained by methanolysis of aminosidine. α and β were added to give concentrations of 48 μg/ml and 82 μg/ml, respectively.

TABLE 4. INFLUENCE OF DNP AND OF AMINOSIDINE ON O_2 UPTAKE BY RAT LIVER MITOCHONDRIA IN DEFICIENCY OF ADP AND OF Pi

Additions	ADP deficiency (μl O_2)	Pi deficiency (μl O_2)
None	116	172
DNP 10^{-4}M	248	314
Aminosidine 0·8–10^{-4}M	96	184
DNP 10^{-4}M + aminosidine 0·8–10^{-4}M	277	268

Substrate: glutamate; incubation time 60 min.

TABLE 5. INLFUENCE OF DNP AND OF AMINOSIDINE ON ATPASE ACTIVITY OF RAT LIVER IN MITOCHONDRIA

Additions	Fresh mitochondria % hydrolysed ATP	Aged mitochondria* % hydrolysed ATP
None	4·9	48
DNP, 10^{-4}M	58	51
Aminosidine, 50 μg/ml	8·5	44

Incubation for 10 min at 30 °C.
* Sucrose suspensions of mitochondria kept for 2 h at 37 °C.

TABLE 6. REVERSAL BY Mg^{2+} OF THE INHIBITING ACTION OF AMINOSIDINE ON P:O OF RAT LIVER MITOCHONDRIA

Expt.	$MgSO_4$ μM/ml	No addition	Aminosidine 50 μg/ml	Inhibition (%)
		P:O	P:O	
No. 1	7·5	2·6	1·64	37
	15	2·9	2·35	21
No. 2	7·5	2·43	1·45	40
	22·5	2·42	2·39	0·8

Substrate: ketoglutarate.

dinitrophenol, does not stimulate ATPase activity of fresh mitochondria. It does not either influence that of aged mitochondria (Table 5). The uncoupling activity of aminosidine is prevented by an excess of Mg^{2+} ions (Table 6). The analysis by titration, according to Albert,[10] did not show formation of complexes of aminosidine with Mg^{2+}.

DISCUSSION

Among the oligosaccharidic antibiotics tested, those having both a tri- or tetrasaccharidic molecule and the character of polyvalent base, act as uncouplers of mitochondrial oxidative phosphorylations. As concerns aminosidine, we could prove, after a comparison with the methanesulphonate derivatives and the products of methanolysis, that both the presence of free amino groups and a certain structural complexity are required for the occurrence of an inhibiting activity on the processes of oxidative phosphorylation. The mechanism of the action of aminosidine differs from that of DNP as demonstrated by the absence of any effect on ATPase activity and by lack of QO_2 stimulation in phosphate, or phosphate acceptor deficient, systems. The same behaviour was observed in the antibiotics belonging to tetracyclines.[13, 14] These antibiotics, too, induce a decrease of the P/O ratio in mitochondria and do not stimulate O_2 uptake in phosphate or ADP-deficient media. Their action is antagonized by Mg^{2+} at concentrations similar to those antagonizing aminosidine.

From the comparison between aminosidine and DNP it may be calculated that while the latter would act previously to the introduction of Pi, thus favouring the hydrolysis of a non-phosphorylated intermediate ($X \sim I$),[15] aminosidine would exert its influence on a reaction in which both ADP and Pi are involved. According to the formulation of Slater and Hulsmann[15] it is possible that this reaction be: $X \sim P + ADP \rightleftharpoons ATP + X$. $X \sim P$ is formed by a Pi requiring reaction: $X \sim I + Pi \rightleftharpoons X \sim P + I$. It is known that this reaction requires Mg^{2+} as coenzyme: therefore we may suppose that aminosidine, in its quality of polyvalent base, acts by removing the Mg^{2+} ions from the enzyme surface. We observe, however, that aminosidine has no effect on the action of hexokinase, a further Mg^{2+} requiring enzyme.

We hope to draw further conclusions from the study of the influence of aminosidine on the partial reactions catalysed by submitochondrial particles.

Vol. 7
1961

REFERENCES

1. F. Arcamone, C. Bertazzoli, M. Ghione and T. Scotti, *G. Microbiol.* **7**, 251 (1959).
2. F. Arcamone and F. Bizioli, *Gazz. Chim. Ital.* **87**, 896 (1957).
3. F. Arcamone. Personal communication.
4. T. H. Haskel, J. C. French, Q. R. Bartz, *J. Amer. Chem. Soc.* **81**, 3480 (1959).
5. G. Hegemann, F. Nomine and L. Penasse, *Ann. Pharm. Franc.* **16**, 585 (1958).
6. S. Umezawa, V. Ito, S. Fukatsu and S. H. Umezawa, *J. Antibiot.* A, **12**, 114 (1959).
7. W. C. Schneider, *J. Biol. Chem.* **176**, 259 (1948).
8. B. B. Marsh, *Biochem. Biophys. Acta* **32**, 357 (1959).
9. H. A. Lardy and H. Wellman, *J. Biol. Chem.* **195**, 215 (1952).
10. D. K. Myers and E. C. Slater, *Biochem. J.* **67**, 558 (1957).
11. S. P. Colowick and H. M. Kalckar, *J. Biol. Chem.* **148**, 117 (1943).
12. A. Albert, *Biochem. J.* **47**, 531 (1950).
13. T. M. Brody, *Proc. Soc. Exp. Biol., N.Y.* **80**, 533 (1952).
14. T. M. Brody, R. Harwitz, J. A. Bain, *Antibiot. and Chemother* **4**, 864 (1954).
15. E. C. Slater, W. C. Hülsman, *Ciba Foundation Symposium on the Regulation of Cell Metabolism* (Edited by G. E. W. Wolstenholme and Cecilia M. O'connor, J. & A. Churchill Ltd, London p. 48) (1959)

Biochemical Pharmacology, 1961, Vol. 7, pp. 169–180. Pergamon Press Ltd., Printed in Great Britain.

THE INFLUENCE OF THIAMINE AND RELATED COMPOUNDS ON THE IRIS OF THE FROG

ARTUR BER and RINA SINGER-ALTBEKER

Department of Endocrinology of the Rogoff Medical Research Institute, Beilinson Hospital, Petah Tiqva, Israel

(*Received* 6 *March* 1961)

Abstract—Injection of thiamine to *Hyla arborea* and *Bufo viridis* causes closing of the iris, and the time of closing is roughly proportional to the dose used. The thiazolic and pyrimidinic components of thiamine are inactive.

Thiamine monophosphate, thiamine diphosphate, pyrithiamine and oxythiamine, act similarly to thiamine, but in higher doses. Acetylthiamine is effective almost in the same dose as thiamine.

Thiamine probably acts directly on the iris, while the other compounds must undergo hydrolysis and free thiamine must be produced in order to show activity.

Thiamine monophosphate in high doses is ineffective and its previous administration inhibits the action of thiamine diphosphate but not of thiamine.

It is supposed that there exist two different thiamine-phosphatases: Th-pyrophosphatase and Th-orthophosphatase, which contain the same or similar coenzymes but different apoenzymes.

INTRODUCTION

IN THE course of our experiments on the influence of vitamins on hormone activity an effect of thiamine (Th) on the iris of normal frogs was observed. As very few observations have been reported on the influence of Th on men and animals when not in a state of avitaminosis or hypovitaminosis, it seemed worthwhile to investigate this phenomenon.

The investigation of di Palma and Hitchcock[1] and Molitor and Emerson[2] deal principally with the toxic effect of high doses of Th in men and animals. The toxic doses given in the literature differ widely, reflecting the difference in the sensitivity of experimental animals as well as the lack of uniformity of the experimental conditions.

Toxic manifestations of Th have been attributed to curare-like activity,[3] to neuromuscular and ganglionic blockade[1] or to paralysis of the respiratory and vascular system.[4] Goldfeld[5] observed an increased visual acuity in healthy men given daily 50 mg Th from 20 to 30 days. It has also been reported that high doses of Th protect men against insect bites,[6] probably due to a particular smell of the sweat of those receiving rich treatment. Neither of these last two reports, however, as far as can be ascertained, has been confirmed.

In addition some pharmacological experiments on isolated organs have been reported. It has been found that Th antagonized acetylcholine activity in isolated frog and tortoise hearts, in rat and rabbit intestine and in virgin uterus of guinea-pig[7–14] but it increased the sensitivity of eserinized leach muscle[15] to this substance. In

avitaminotic pigeons the sensitivity to acetylcholine is decreased.[9] Furthermore, Th inhibits cholinesterase activity[3, 16] and antagonizes the effect of nicotin on isolated smooth muscles. This last effect is not specific for Th, as other substances have been reported as having the same influence.[17–20]

In the reported studies no specific action of Th in healthy animals has been noted.

The present paper presents our observations on myosis in frogs induced by Th and some chemically related compounds. The physiological aspect of this investigation will be the subject of a separate paper.

MATERIAL AND METHODS

Two species of frogs, *Bufo viridis* and *Hyla arborea*, were used after our tests with mammals (albino and wild rats, white and black mice, rabbits, dogs, cats and cows) showed no visible reaction to Th administration. Freshly caught frogs were put in individual glass vessels and kept in a cool place. No food was supplied. Their body weight varied from 5 to 25 g, but in each experimental series frogs of approximately equal weights were used. Each animal was numbered and the course of treatment was registered individually. Animals used more than once, were given a minimum of 3-days rest between experiments, and frogs showing any disturbances were eliminated.

The following substances were tested. Thiamine* (Th), thiazolic component of Th*, pyrimidinic component of Th*, oxythiamine* (Oxy-Th), thiamine pyrophosphate* (ThDP), thiamine monophosphate† (ThMP), acetyl thiamine† (Acet-Th), and pyrithiamine‡ (Pyri-Th). Doses varied from 3·75 to 60 mg/10 g body weight for each compound when injected to *Bufo*, while *Hyla* received from 1 to 16 mg/10 g body weight. In addition we experimented with acetylcholine§ (ACh) (0·001–2 mg/10 g body weight for both species), ATP‖ (2–8 mg/10 g body weight for both species), sodium sulphathiazol** (10–150 mg/10 g body weight for *Bufo*), physostigmine* (0·01–2 mg/10 g body weight for both species), KCl, KH_2PO_4 (1–20 mg/10 g body weight for *Bufo*) and HCl and NaOH diluted with saline to obtain a pH range of 1–10.

All substances were dissolved in saline or water and injected through the lymphatic femoral sac into the dorsal lymphatic sac. The volume of the solution injected did not exceed 0·5 ml. After injection the size of the pupil was observed for any change occurring.

The pupil of frogs, kept under normal daylight conditions, is oval in shape, and is denoted throughout this paper as 50 per cent opening. The maximal extension which is circular and which can be induced by the administration of adrenaline or noradrenaline is described as 100 per cent opening. Under the influence of contracting substances the iris becomes gradually smaller reaching finally the size of a thin line. This final state is referred to throughout this paper as a 0 per cent opening and considered to be a positive reaction (Fig. 1).

The size of the iris was evaluated approximately and without the aid of instruments. During the long course of experiments we acquired skill in evaluation to such a

* Hoffman La Roche, Basle (Switzerland).
† NBC, Cleveland (Ohio).
‡ Sigma, St. Louis (Missouri).
§ Assia, Ramath Gan (Israel).
‖ Light & Co. Ltd, Colnbrook Bucks (England).
** Boots, Nottingham (England).

Vol. 7
1961

ol. 7
961

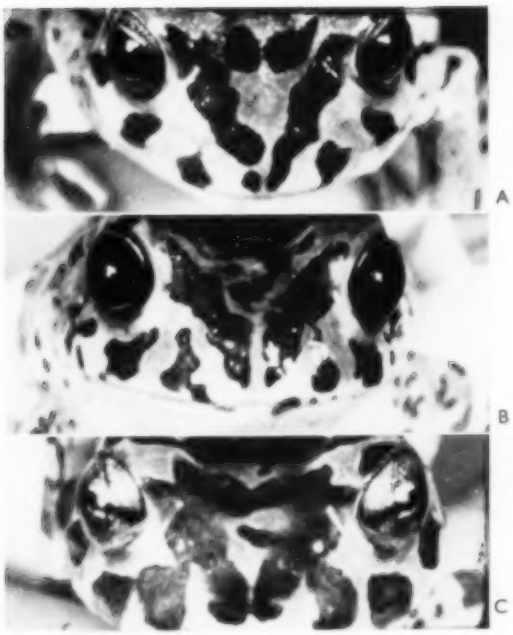


FIG. 1. A. Eyes of untreated *Bufo*. B. Eyes of *Bufo* after injection of adrenaline. C. Eyes of *Bufo* after injection of Th.

facing page 170

BLANK
PAGE

Vol. 7
1961

degree, that similar estimates were obtained by different workers. Deviations, however, in the estimation of the intermediate sizes of the pupil (up to a maximum of 10 per cent) did occur.

The period which elapsed between the instant of injection of the test substance and the maximal closing of the iris is referred to as "time of closing".

The treated animals were kept under observation for 2 hr, and, in some instances, for a longer period. It often happened that the time of closing was not the same for both eyes in which case the mean value is given.

RESULTS

The results of the experiments in which Th and related substances were administered to *Bufo* and *Hyla* are shown in Table 1. Wide variations in the reaction speed were observed within the same species, even after administration of equal doses of the same preparation. There were also cases in which the same animal, when used more than once, reacted differently to the same treatment (Table 2). The difference in the time of closing of the two irises generally did not exceed 2 min, although, in rare instances, a difference of up to 20 min was noted. No prevalence of sensitivity of the left or right eye was noted.

It must be pointed out that in summer the time of reaction was much shorter than in winter. So far, we have not been able to explain this phenomenon. Although experiments were performed around the year, the results reported below relate to the period May to October (late spring and summer). The findings in other seasons show parallel but not identical trends. For a controlled study all experiments should be performed simultaneously.

All Th injections, in doses not lower than 7·5 mg/10 g body weight for *Bufo* and 2 mg/10 g body weight for *Hyla*, resulted in closing of the iris, when conditions of illumination were usual. The period during which the iris remained closed, ranged from 1 to 12 hr or more in some cases and depended on the dose. The time of closing of the iris was in reverse proportion to the dose administered (Table 1 and Fig. 2), which correlation is statistically significant. When using doses smaller than the above, not all frogs reacted and the number of the animals which did not respond increased with decreasing dose.

When the experiments were conducted in the dark, in which case the pupil reaches its maximal opening, the administration of Th reduced the size of the pupil up to approximately 50 per cent but no further.

Doses higher than 7·5 mg/10 g body weight in *Bufo* and than 3 mg/10 g body weight in *Hyla* were toxic and caused paralysis, increasing in severity with the amount injected and finally causing death. Such observations were already reported by other investigators.[1, 2] Similar toxic manifestations were also observed after injection of compounds chemically related to Th. Pigmentation changes were also brought about by these compounds, but this will be the subject of separate work.

A study of the components of Th separately revealed that the pyrimidinic component had no influence on the iris of either species, while the thiazolic component brought about myosis in *Bufo* only, but in a very irregular manner. When the two components were given simultaneously the action of either was not influenced by the other.

TABLE 1. EFFECT OF ADMINISTRATION OF TH AND CHEMICALLY RELATED SUBSTANCES ON FROG IRIS

Material	*Bufo*						*Hyla*					
	Number of animals	Dose mg per 10 g body weight	% of react. animal	Time of closing (min)			Number of animals	Dose mg per 10 g body weight	% of react. animal	Time of closing (min)		
				Min.	Max.	Aver.				Min.	Max.	Aver.
Vitamin B_1	13	3·75	77	8	33	19·4	20	1	50	5	17	9·2
	14	7·5	100	5	16	9·8	15	2	100	3	16	8·5
	15	15	100	3	12	6·5	15	4	100	2	11	7·1
	15	30	100	2	15	4·9	14	8	100	1	9	4·5
	9	60	100	2	10	3·6	12	16	100	1	5	2·9
4-Oxythiamine chlorid-hydrochlorid	6	3·75	0	—	—	—	8	1	0	—	—	—
	8	7·5	62·5	8	20	11·6	8	2	50	11	19	15·9
	7	15	71·4	5	15	10·6	8	4	100	5	15	8·8
	11	30	100	4	19	9·1	7	8	100	3	15	6·3
	7	60	100	3	11	6·3	8	16	100	1	7	2·9
Acetylthiamine	14	3·75	71·4	7	37	21·3	5	1	20	—	—	—
	13	7·5	92·3	12	60	21·6	14	2	64·4	5	38	16·1
	9	15	100	3	18	8·6	10	4	100	7	27	13·1
	8	30	100	1	9	3·8	10	8	100	3	23	12·6
	5	60	100	1	3	2·2	9	16	100	1	8	5
Thiamine monophosphate	4	3·75	25	—	—	—	5	1	0	—	—	—
	9	7·5	100	8	52	30·7	5	2	40	21	83	52
	11	15	100	7	65	33	10	4	80	7	42	25
	11	30	63·6	4	120	45·5	10	8	100	16	59	35·3
	6	60	0	—	—	—	5	16	100	4	31	19
Thiamine diphosphate	5	3·75	—	—	—	—	8	1	0	—	—	—
	7	7·5	42·9	33	53	44	13	2	61·5	16	30	22·6
	9	15	66·7	21	96	49·3	14	4	71·4	6	33	20·4
	10	30	70	13	110	28	11	8	100	8	32	19·6
	8	60	87·5	8	37	21·5	6	16	100	9	37	17·2

When the animals were previously injected with the pyrimidinic or the thiazolic compound, or with both, Th produced results similar to those observed in the control animals treated with Th alone.

After injection of ThDP, Acet-Th, Oxy-Th and Pyri-Th the rate of closing was proportional to the doses administered (Table 1 and Fig. 2).

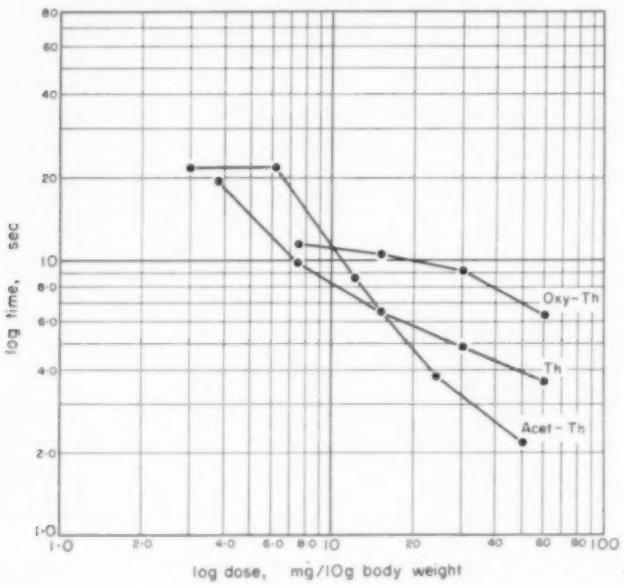


FIG. 2. Log time–log dose relationship of iris closing time in *Bufo* after injection of three different compounds. The amounts given in the curves correspond to the molecular weight of Th.

TABLE 2. VARIATION IN IRIS CLOSING TIME AFTER REPEATED INJECTIONS OF TH (7·5 MG/10 G BODY WEIGHT) TO THE SAME FROGS

Date	Time of iris closing (min)		
	Bufo 1	*Bufo* 2	*Bufo* 3
10.9.59	12	11	9
13.9.59	2	—	33
17.9.59	10	13	17
21.9.59	6	8	20

A dose of 1 mg/10 g body weight of pyrithiamine given to *Hyla* still brought about the closing of the iris, while 0·5 mg/10 g body weight, had no effect. Unfortunately, our supply of Pyri-Th was very limited and we could not perform a complete series of experiments, but we were able to show a correlation between the injected dose and the time of closing of the iris. In the case of Oxy-Th this correlation was clearly evident

as seen in Table 1 and Fig. 2. When this compound was injected simultaneously with Th the time of the reaction was shorter than anticipated from the sum of the two compounds.

ThMP was effective but no correlation was found between the dose and the speed of action. A dose of 60 mg/10 g body weight of this compound had no effect on *Bufo*, while in *Hyla* the reaction was irregular. In *Bufo* injections of ThMP in high doses which had no effect on the size of the pupil did not alter the action of Th injected simultaneously or after 10–15 min. On the other hand ThDP when injected after previous administration of high doses of ThMP proved to be ineffective.

Administration of ACh, physostigmine or both simultaneously, KH_2PO_4 or KCl dissolved in saline or water had no influence on the iris. High doses of potassium salts caused paralysis in frogs notably in the rear limbs and subsequent death. ATP administered alone or with either pyrimidinic or thiazolic compound, or with both, had no effect on the iris of frogs. Sodium sulphathiazole and diluted solutions of HCl and NaOH at pH ranging from 1 to 10, also had no influence.

DISCUSSION

The main purpose of the present study was to determine whether the specific action of Th on the frog iris was direct or through activation of other substances capable of influencing the size of the pupil of frogs. We found that ACh and physostigmine, which are known to exert an influence on mammalian eyes, were without effect on frog eyes when administered simultaneously. Potassium salts, found by us to be potent in inducing myosis of isolated frog eyes, had no effect *in vivo* even when doses causing paralysis of the limbs were used. ATP known as energy source for physiological Th activity did not show any effect on the frog iris.

Most of the solutions examined were acidic, but injections of HCl and NaOH with pH's ranging from 1 to 10 were ineffective, showing that the pH did not play a significant role. We were led to assume therefore that the myosis was effected directly by Th, or possibly by one or more substances derived from it.

Neither of the components of Th, the pyrimidinic or the thiazolic could be acknowledged as the active substance since the former did not show any activity, while the latter acted only on *Bufo* and in quantities much larger than Th. In addition the effect of these compounds was not correlated with the dose.

Th is known to inhibit the action of nicotine on isolated smooth muscle of the intestine of rabbits, frogs and guinea-pigs. A similar effect of the thiazolic component of Th, as well as of sulphathiazole has been reported.[17–20] In our experiments, however, no action of the sodium salt of sulphathiazole on the iris was noted.

Administration of both thiaminic components simultaneously, whether in the presence or absence of ATP, had also no effect on the iris and all above-mentioned substances were unable to inhibit the activity of Th injected simultaneously or shortly after.

It may be concluded therefore that the complete molecule of Th is required to obtain thorough closing of the iris. Consequently it may be assumed that *Hyla* is unable to synthesize Th from its components. On the other hand such a synthesis could take place at a low rate in the case of *Bufo* as seen from the weak and irregular action of the thiazolic component in the latter. However, this is questionable as in

Vol. 7
1961

Bufo the pyrimidinic component did not promote the activity of the thiazolic component.

It may be supposed that in order to obtain activity, both components have to be attached to a reacting centre (enzyme protein? muscle protein?) and that the methylenic bridge between the two components is essential, since simultaneous administration of both components did not cause a positive reaction. One possibility would be, that the reacting centre tends to be linked to the molecule of Th, at least at three points, i.e. one at the thiazolic component, one at the connecting methylenic bridge and one at the pyrimidinic component.

Another possibility is, that the reacting centre tends to be linked solely to the two components of Th, the methylenic bridge serving only as a factor conditioning their spatial arrangement. None of these links would be very strong, since previous administration of both components did not inhibit the action of Th. The latter probably expels the linked compounds from their complexes with the reacting centre, and takes over, forming much stronger links. Such a mechanism would be analogous to the action of ACh upon active centre of intracellular protein, as described by Nachmanson and Wilson[21]. The comparison seems permissible since both Th and ACh belong to the group of biologically very active "onium" compounds, which are derivatives of quaternary ammonium salts.

The possibility also exists that Th does not act directly upon the reacting centre but that it produces enzymatically another active factor whose components x, y are first linked at the appropriate place to the Th molecule (x–Th–y), then linked with each other and finally separated from their complex with Th in the form x–y, which would be the active compound. The role of the methylenic bridge in this case would be to provide the necessary spatial arrangement for the binding of x and y with each other after linking to the components of Th. This pathway would resemble the model of hydrolysis of glucose by glucosidase as suggested by Pigman[22].

The generally accepted view is that the main and perhaps the only role of Th is its inducement of the active cocarboxylase, and only few investigators[23–30] attribute some activity to free Th itself. In our observations the reactivity to ThDP was much lower than to Th when administered in corresponding amounts (Table 1 and Fig. 2). The difference in dose required to produce the same time of closing excludes the possibility that Th acted through its pyrophosphate derivative. The opposite is more probable, i.e. that the degradation of ThDP to Th is necessary in order to obtain positive results. Possibly the pyrophosphate chain prevents joining of the molecule to the reacting centre.

The possibility, however, cannot be excluded that the Th molecule is able to penetrate the cell more easily than ThDP and therein undergo conversion to ThDP, which last derivative may be the active factor. To solve this problem we examined compounds analogous to Th, namely Pyri-Th and Oxy-Th which are known to antagonize Th in animals (e.g. mice) and yeasts. Their mode of action is not definitely known, but the accepted view is that Pyri-Th antagonizes Th itself, while Oxy-Th is rather an antagonist of ThDP. Woolley[26] postulated that Pyri-Th together with pyrophosphate and a specific enzyme form a stable complex. In this way the enzyme becomes blocked and is not available for the synthesis of ThDP from Th. Oxy-Th acts in a similar way, but its pyrophosphate is easily separated from the enzyme

complex. Oxy-Th-pyrophosphate would bind with apocarboxylase making the synthesis of holo-carboxylase from cocarboxylase impossible (Fig. 3).

Some investigators (Muralt, for example), on the basis of these assumptions, tried to establish whether some specific pharmacological reactions were due to Th or to its pyrophosphate. In our experiments neither Pyri-Th nor Oxy-Th inhibited the action of Th or ThDP on the closing of the iris. Moreover, they attributed an activity of their own as seen in Table 1 and Fig. 2.

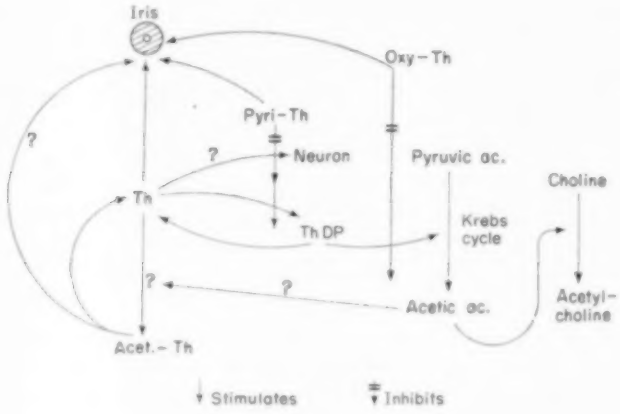
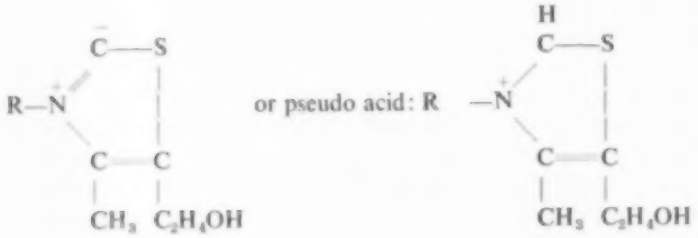


FIG. 3. Some pathways of the supposed action of Th and related compounds. For explanation see the text.

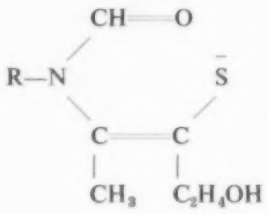
The analogy found in the activity of Th, Pyri-Th and Oxy-Th is not surprising, since it is known, that substances with related chemical structures and steric configurations (isosteric compounds) reveal similar physiological activity. This is encountered in the case of benzenic and thiophenic, thiazolic and pyridinic compounds. As a matter of fact Pyri-Th was originally synthesized for the purpose of obtaining a compound with an activity analogous to Th and not antagonistic to it.[31]

The active group in the process of decarboxylation of α-keto acids is probably a bipolaric ion:[32]



Vol. 7
1961

We suppose that in our experiments also these groups are the active ones, or at least partially so. We base this on the fact that substitution of the thiazolic ring by a pyridinic one, as in the case of Pyri-Th, does not affect the specific physiological activity. This obviously, could happen, if the thiazolic component acted in the form of the thiolic pseudobase:



The fact that Pyri-Th and Oxy-Th are active in inducing myosis in frogs similarly to Th, speaks for a different mechanism than the one involved in the known process of Th activities. The antagonistic action of these antimetabolites apparently involve receptors other than those related to the influence on the iris (Fig. 3).

It should be noted that these compounds inhibit also the action of ACh on isolated frog heart, similarly to Th and that Oxy-Th potentiate the action of Th,[14] as also found in the present study.

The results obtained on administration of ThMP are of special interest. This compound acts much slower (in winter) than Th and ThDP and augmentation of its dose not only does not increase the speed of the reaction, but from a certain level upwards the reaction does not occur at all (especially in *Bufo*). Doses higher than 60 mg/10 g body weight were ineffective in *Bufo*, while in *Hyla* the increase of the dose caused an irregular response. Probably ThMP, in high doses, inhibits the activity of the phosphatase which converts ThMP to Th. Many cases of non-competitive inhibition of enzymes caused by excess of substrate concentration are known in enzymology, e.g. inhibition of invertase by excess of fructose, β-glucosidase by excess of glucose, urease by excess of urea, or cholinesterase by excess of ACh.[33, 34]

The difference in behaviour between ThDP and ThMP points to the probable existence in frogs and perhaps in other animals of two different enzymes, namely Th-orthophosphatase and Th-pyrophosphatase, the last being much more active.

Preliminary experiments with products of incubation of ThDP and ThMP with blood of rats (known to be rich in phosphatases), injected into frogs, were made. It was found that when ThDP was used, the time of closing of the iris was much shorter than when no incubated material in the same dose was injected. It almost corresponded to the time calculated from the time–dose relationships when the same amount of the free Th, as contained in ThDP, was injected. In contrast, incubation of ThMP did not alter its activity. This problem needs more additional work and will be investigated separately, but it may be pointed out that ThMP in acidic medium containing myosin, is much more stable than ThDP or ThTP, as described in the work of Greiling and

Kiesow[35]. There is probably a correlation between this and the weak activity of ThMP hydrolyzing enzymes.

It was found that previous administration of high inactive doses of ThMP to *Bufo* did not change the action of Th, while ThDP injected under the same conditions was completely inactive. It may be concluded from these results, that the unhydrolyzed ThMP does not bind the active centre, or if it does, such a link is very weak so that Th may easily replace it.

The lack of reaction to ThDP after previous administration of ThMP, is indicative, that excess of circulating ThMP inhibits not only the enzyme which hydrolyses ThMP to Th, but also the enzyme which hydrolyses ThDP to Th. One possible explanation is that both enzymes possess a common coenzyme but different apoenzymes. It cannot be excluded, however, that the same enzyme acts on both compounds and that hydrolysis of the enzyme–ThMP complex is much slower than that of the enzyme–ThDP. It may be concluded, whether there are one or two thiamine-phosphatases, that there is a chemical competition between ThDP and ThMP for the hydrolysing enzyme (or enzymes); therefore a anticocarboxylase action of ThMP possibly exists under certain conditions. Of course the blackade of the enzyme would not affect the action of Th, a fact proved by us experimentally.

In contrast to ThMP, Acet-Th was found to be as active as free Th. It may be supposed that it undergoes a very rapid hydrolysis, releasing active free Th or that, unlike Th-phosphates, it binds the active centre in the unchanged form. Probably the acetyl group does not affect the ability of the molecule to become linked to the reacting centre, as it is known that the ability of molecules to bind the proper receptors, greatly depends on their steric configuration and on their rigidity or elasticity.

One of the main roles of carboxylase is to enable the splitting of pyruvic acid, liberating acetate which participates in the synthesis of ACh. It cannot be, however, excluded, that Th undergoes acetylation simultaneously and that Acet-Th participates in some metabolic processes. This supposition is supported by the work of Kuhn *et al.*[36], who attributed to Acet-Th a role similar to ACh in nerve metabolism.

The present observations indicate that Th is able to participate in active metabolism in frogs, independently from cocarboxylase. Support for this assumption may be found in some investigations dealing with experiments *in vitro*. Ochoa[37,38] observed that free Th stimulated the decarboxylation of pyruvic acid by alkaline-washed yeasts in the presence of an excess of cocarboxylase, and methods for separate determination of Th and ThDP were based on this observation. Westerbrink[39] showed that Acet-Th acts similar to Th although to weaker degree (50 per cent). Recent experiments[40, 41] have shown that free Th is able to catalyse the decarboxylation of α-keto acids in the absence of other enzymes, though to a much less extent than ThDP.

Experiments performed on vertebrates *in vivo* are less convincing. Woolley and Merrifield,[29,30] as well as Muralt,[25] basing their conclusions on results obtained by using Oxy-Th and Pyri-Th in rats and mice, believe that Th has a direct specific action on the nervous system not mediated through ThDP (Fig. 3). On the other hand, experiments of Caro *et al.*[42, 43] demonstrate that Oxy-Th and Pyri-Th have a similar action, the first decreasing the amount of Th mainly in muscles and liver, the second decreasing the amount mainly in the brain. Correspondingly, clinical manifestations, when using both compounds, are quite different. Di Palma and Hitchcock[1] attributed the difference

Vol. 7
1961

in action between Oxy-Th and Pyri-Th to the fact that Pyri-Th is much more active in causing neuromuscular and ganglionic blockade than Oxy-Th and not to their influence on Th activity.

It may be concluded that the presently observed *in vivo* action of Th and possibly also of Acet-Th, is not related to its classic metabolic role. The mode of action seems to be direct and not mediated through cocarboxylase. The later acts probably after undergoing hydrolysis to produce free Th (Fig. 3).

The question whether Th is involved in the physiological regulation of the size of the pupil in the frog, as yet cannot be answered, and will be a subject of further investigation.

Acknowledgement—The help of Mr. D. Allalouf is greatly appreciated.

REFERENCES

1. J. R. di Palma and P. Hitchcock, *Anesthesiology* **19**, 6, 762 (1958).
2. H. Molitor and G. A. Emerson, *Vitam. & Horm.* **6**, 69 (1948).
3. J. Cheymol, F. Bourillet, C. Levassort and L. Kerp, *Arch. Int. Pharmacodyn.* **111**, 36 (1957).
4. J. A. Smith, P. P. Foa and H. R. Weinstein, *Fed. Proc.* **6**, 204 (1947).
5. N. G. Goldfeld, *Prob. Fisiol. Opt.* **11**, 66 (1955).
6. Monatsspiegel. *Redaktionsartikel* **7**, 7, 163 (1958).
7. R. Agid and J. Balkanyi, *C.R. Soc. Biol., Paris* **127**, 680 (1938).
8. H. J. Bein, *Helv. Physiol. Acta* **5**, 190 (1947).
9. V. Erspamer, *Arch. Int. Pharmacodyn.* **63**, 4, 385 (1939).
10. V. Erspamer, *Arch. Ital. Sci. Farmacol.* **2**, 1 (1940).
11. E. Gautier, *Helv. Physiol. Acta* **8**, 2, 195 (1950).
12. E. Gautier. Thèse Univ. de Berne (1950).
13. F. Gross, *Helv. Physiol. Acta* **5**, 122 (1947).
14. H. P. Gurtner, *Pflüg. Arch. ges. Physiol.* **265**, 423 (1958).
15. L. Binet and B. Minz, *C.R. Soc. Biol., Paris* **117**, 1029 (1934).
16. H. N. De, *J. Med. Res.* **43**, 1, 71 (1955); cited from *Biol. Abstr.* 31106 (1956).
17. H. Haimovici and E. P. Pick, *Proc. Soc. Exp. Biol., N.Y.* **62**, 234 (1946).
18. E. P. Pick, J. W. Brooks and K. Unna, *J. Pharmacol.* **81**, 133 (1944).
19. E. P. Pick and K. Unna, *J. Pharmacol.* **87**, 138 (1946).
20. K. Unna and E. P. Pick, *J. Pharmacol.* **81**, 294 (1944).
21. D. Nachmansohn and I. B. Wilson, *Advanc. Enzymol.* **12**, 259 (1951).
22. W. W. Pigman, *Chemistry of the Carbohydrates*. Academic Press, New York (1948).
23. A. Muralt, *Mitt. Naturf. Ges. Bern* **14**, 97 (1957).
24. A. Muralt, *Exp. Cell. Res.* Suppl. **5**, 72 (1958).
25. A. Muralt, *Neue Ergebnisse der Nervenphysiologie*. Julius Springer, Berlin (1958).
26. D. W. Woolley, *A Study of Antimetabolites*. John Wiley, New York (1952).
27. D. W. Woolley, *Bull. Soc. Chim. Biol., Paris* **36**, 1207 (1954).
28. D. W. Woolley, *Experientia* **13**, 165 (1957).
29. D. W. Woolley and R. B. Merrifield, *Fed. Proc.* **11**, 458 (1952).
30. D. W. Woolley and R. B. Merrifield, *Bull. Soc. Chim. Biol., Paris* **36**, 1207 (1954).
31. A. H. Tracy and R. C. Elderfield, *J. Org. Chem.* **6**, 54 (1941).
32. R. Breslow. Cited from R. G. Yount and D. E. Metzler, *J. Biol. Chem.* **234**, 4, 738 (1959).
33. J. Kühnau, Antagonismen und Konkurenzen um den Platz am Ferment. *Biologie und Wirkung der Fermente*. Julius Springer, Berlin (1953).
34. J. B. Sumner and G. F. Somers, Influence of substrate concentration. *Chemistry and Methods of Enzymes*. Academic Press, New York (1950).
35. H. Greiling and L. Kiesow, *Z. Naturf.* B **12**, 605, 672 (1957).
36. R. Kuhn, Th. Wieland and H. Huebschmann, *Z. physiol. Chem.* **259**, 48 (1939).

37. S. OCHOA and R. A. PETERS, *Biochem. J.* **32**, 1501 (1938).
38. S. OCHOA, *Nature, Lond.* **141**, 831 (1938).
39. H. G. WESTERBRINK, *C.R. Lab. Carlsberg* **23**, 14, 195 (1940).
40. E. YATEO-MANZO, F. RODDY, R. G. YOUNT and D. E. METZLER, *J. Biol. Chem.* **234**, 4, 733 (1959).
41. R. G. YOUNT and D. E. METZLER, *J. Biol. Chem.* **234**, 4, 738 (1959).
42. L. DE CARO, G. RINDI and E. GRANA, *Experientia* **10**, 3, 140 (1954).
43. L. DE CARO, G. RINDI, V. PERRI and G. FERRARI, *Experientia* **12**, 8, 300 (1956).

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 181–186. Pergamon Press Ltd., Printed in Great Britain.

EFFECT OF L-DOPA AND N-METHYL-N-BENZYL-2-PROPYNYLAMINE.HCl ON DOPA, DOPAMINE, NOREPINEPHRINE, EPINEPHRINE AND SEROTONIN LEVELS IN MOUSE BRAIN

R. G. WIEGAND and J. E. PERRY

Chemical Pharmacology Section, Division of Experimental Therapy, Abbott Laboratories, North Chicago, Illinois

(*Received* 27 *December* 1960)

Abstract—A procedure for the determination of DOPA, dopamine, norepinephrine, epinephrine and serotonin in a single tissue homogenate is given. N-Methyl-N-benzyl-2-propynylamine.HCl (A19120, MO-911) causes increased dopamine, norepinephrine and serotonin levels in mouse brain. Dopamine levels return to normal within 48 hr, at which time the levels of norepinephrine and serotonin are still elevated. L-DOPA causes increased DOPA, dopamine and norepinephrine concentrations, decreasing in this order. After MO-911 followed by L-DOPA, the increases in DOPA, dopamine and norepinephrine are greater than with either drug alone or the sum of these increases. At the time of sacrifice, after the administration of MO-911 and L-DOPA, the mice were markedly hyperactive, but the relative contribution of the increases in DOPA, dopamine, norepinephrine or serotonin to this activity could not be determined.

N-METHYL-N-BENZYL-2-PROPYNYLAMINE.HCl (A19120, MO-911) is a potent non-hydrazide monoamine oxidase (MAO) inhibitor.[1] A dose of 100 mg/kg intraperitoneally causes no behavioral changes in mice, but subsequent to the further intraperitoneal administration of 200 mg of DL-dihydroxyphenylalanine (DL-DOPA) per kg a marked increase in activity and aggressiveness is obtained.[2, 3] This dose of DL-DOPA alone does not cause a change of activity in control mice.[4]

Bertler and Rosengren[5] report increased activity correlated with increased brain dopamine levels in rabbits given 600 mg of DOPA per kg. Carlsson *et al.*[6] also found increased dopamine and norepinephrine levels in mouse brain 6 hr after monoamine oxidase inhibitors. This suggests that the activity of mice given an inhibitor of MAO followed by DOPA may be due to increases in dopamine or norepinephrine. The relative increases in these amines and serotonin may be different after a MAO-inhibitor alone, and the duration of the increases may also differ. To investigate the changes in the concentration of these and related compounds, a new separation procedure for the determination of DOPA, dopamine, norepinephrine, epinephrine and serotonin in a single brain homogenate was developed and is included as part of this report.

METHODS

MO-911 dissolved in saline and L-DOPA (Calif. Corp. Biochem. Res., CfP grade) suspended in 0·5% Methocel were injected intraperitoneally, 0·02 ml/g body weight.

The concentration of MO-911 was corrected for HCl so that all doses refer to free base. White male mice in the weight range from 19 to 28 g were used.

Three pooled mouse brains were homogenized in 2 vols. of 0·01 N HCl and extracted by the method of Shore and Olin[7], using 3 ml of homogenate, 2 g of NaCl and 25 ml of *n*-butanol reagent. The second extraction was from 20 ml of the *n*-butanol with 20 ml *n*-heptane into 3 ml of 0·01 N HCl. In each extraction the tubes were shaken at 250 counts/min for 5 min. Aliquots of 0·6 ml of this final acid extract were analyzed for norepinephrine and epinephrine by using pH 5 and pH 3 buffer.[7] Fluorescence was measured in an Aminco spectrophotofluorometer at the uncorrected wavelengths of 397 mμ and 508 mμ.

Of the acid extract, 1·0 ml was placed on a water-washed Dowex 50W-x4 (200–400 mesh) Na-form column, 2 mm high and 5 mm wide. The sample was washed on with a single application of 2 ml of water. The DOPA was eluted in one tube with 8 ml of 0·025 M sodium phosphate buffer, pH 6·5, and the dopamine was eluted in a second tube with 8 ml of the same buffer containing 1 M KCl. This separation of DOPA and dopamine differs from that given by Bertler *et al.*[8] in that the acid extract is placed directly on the column without neutralization and elution is performed at the pH necessary for the development of fluorescence (pH 6·5), rather than with 1 N HCl; thus, two pH adjustments are eliminated. The procedure of Carlsson and Waldeck[9] was used to determine DOPA and dopamine concentrations in the eluates. Because fluorescent intensity developed slowly in these samples unless they were irradiated,[9] fluorescence was determined 22 hr later at the uncorrected activation and fluorescent wavelengths of 334 mμ and 380 mμ, respectively.

The remainder of the acid extract was used for analysis of serotonin by measuring fluorescence of the extract directly at the uncorrected wavelengths of 291 mμ and 342 mμ. It was unnecessary to adjust the acid concentration to 3 N[10] to obtain adequate sensitivity relative to the blank. The reading in 0·01 N HCl at the above wavelengths was thirteen times as sensitive as the reading at the peak at 545 mμ in 3 N HCl.

An acid blank was run with each experiment. Standard solutions of L-DOPA, dopamine, norepinephrine, epinephrine and serotonin in 0·01 N HCl were added in volumes of 0·01 to 0·04 ml to 3-ml aliquots of brain homogenate and a 3-ml aliquot of the same homogenate was analyzed as a control. The corrected fluorescent intensity (meter multiplier $\times$ $\%T$ corrected for that of the blank) of all compounds was linear in the concentration range reported. Standard solutions of dopamine, norepinephrine and serotonin in 0·01 N HCl were carried through the final color development with each set of samples analyzed.

The specificities of the procedures for each amine are not all absolute, and the contributions to the readings in each procedure due to the presence of other amines are given in Table 1. These contributions were determined by analyzing standard solutions containing one of the amines, 10 μg of free base per 3 ml of 0·01 N HCl, by the complete procedure, including extraction. Contributions of less than 1 per cent are not significantly different from blank values. The data in Table 1 show that the only significant contribution to the DOPA procedure is from dopamine, probably arising from leakage of the dopamine from the column during DOPA elution. The dopamine procedure is not influenced by large excesses of any of the other amines. Norepinephrine determinations are predominantly influenced by dopamine and epinephrine, the dopamine being the greater factor due to its greater concentration

in control and treated brain. The correction of the number of micrograms of norepinephrine by 2·4 per cent of the micrograms of DOPA present is not necessary in mouse brain, unless DOPA has been administered. Epinephrine is determined with only a slight contribution from norepinephrine. The serotonin procedure involves significant contributions to the fluorescence due to dopamine, norepinephrine and epinephrine. However, the quantity of epinephrine present in mouse brain is so small that the correction can be ignored, and the corrections for dopamine and norepinephrine are considerable only when concentrations of these amines are elevated.

TABLE 1. SPECIFICITY OF THE PROCEDURES FOR DETERMINATION OF DOPA, DOPAMINE, NOREPINEPHRINE, EPINEPHRINE AND SEROTONIN

Amine	Contributions of amines to the readings in each procedure, given as per cent of the weight of free base of contributing amine present*				
	DOPA procedure	Dopamine procedure	Nor-epinephrine procedure	Epinephrine procedure	Serotonin procedure
DOPA	100·0	0·8	2·4	0·6	0·8
Dopamine	5·8	100·0	8·0	0.0	3·0
Norepinephrine	−0·7	−0·3	100·0	1·4	2·6
Epinephrine	−0·2	0·1	57·0	100·0	2·8
Serotonin	−0·3	0·3	0·4	0·3	100·0

* Each value represents the mean of from two to six determinations.

The contributions of interfering amines listed in Table 1 must be made by calculating the correction on the basis of micrograms of interfering amine present. Thus, the norepinephrine should be corrected for dopamine and epinephrine before its level is used in correcting serotonin. Further correction of serotonin and DOPA for dopamine completes all the necessary corrections. The data have also been analyzed by solving five simultaneous equations on an IBM 1620 digital computer, using all the correction factors listed in Table 1. The values for micrograms of amine per gram of tissue obtained by the two methods of calculation do not differ appreciably.

RESULTS AND DISCUSSION

The concentrations of DOPA, dopamine (DA), norepinephrine (NE), epinephrine (EP) and serotonin in brain of control mice and mice treated with 200 mg of L-DOPA/kg 30 min before sacrifice, or with 100 mg MO-911/kg 24 or 48 hr before sacrifice, or with both drugs at the same dose and time before sacrifice, as was used for each drug alone (24 hr for MO-911), are given in Table 2. No changes in behavior were noted in the mice given MO-911. The mice which received only L-DOPA exhibited slightly increased activity when handled, but no changes were observed otherwise. The mice given MO-911 followed by L-DOPA were hyperactive, irritable and aggressive at the time of sacrifice.

The changes in DOPA, DA, NE and EP mouse brain concentrations during the first 24 hr after administration of 100 mg of MO-911/kg intraperiteoneally are shown in Fig. 1. Each value in Fig. 1 is the average of seven determinations, except for some control and 24-hr values, as given in Table 2.

Table 2. Concentration of DOPA, dopamine, norepinephrine, epinephrine and serotonin in whole mouse brain

Treatment	Activity	Mouse brain conc. (μg/g $\pm$ s.d.) (no. of detns.)				
		DOPA	Dopamine	Norepinephrine	Epinephrine	Serotonin
Controls	normal	0·08 $\pm$ 0·09 (12)	0·87 $\pm$ 0·16 (12)	0·36 $\pm$ 0·05 (10)	0·04 $\pm$ 0·01 (13)	0·82 $\pm$ 0·02 (3)
MO-911, 100 mg/kg, −24 hr	normal	0·11 $\pm$ 0·05 (7)	1·03 $\pm$ 0·07 (7)	0·62 $\pm$ 0·05 (6)	0·04 $\pm$ 0·01 (7)	1·14 $\pm$ 0·10 (8)
MO-911, 100 mg/kg, −48 hr	normal	0·09 $\pm$ 0·06 (7)	0·83 $\pm$ 0·03 (7)	0·55 $\pm$ 0·02 (7)	0·03 $\pm$ 0·01 (7)	1·16 $\pm$ 0·03 (7)
L-DOPA, 200 mg/kg, −30 min	slight hyperactivity	0·75 $\pm$ 0·24 (7)	2·80 $\pm$ 0·52 (7)	0·47 $\pm$ 0·06 (7)	0·04 $\pm$ 0·02 (7)	0·72 (2)
MO-911, 100 mg/kg, −24 hr L-DOPA, 200 mg/kg, −30 min	marked irritability	4·38 $\pm$ 0·44 (7)	9·47 $\pm$ 0·60 (7)	0·81 $\pm$ 0·15 (6)	0·04 $\pm$ 0·02 (7)	1·42 $\pm$ 0·05 (3)

The increase in the levels of dopamine, norepinephrine and serotonin in the brain after hydrazide type MAO-inhibitors is well documented, and also occurs with MO-911, an MAO-inhibitor of the propynylamine type (Table 2, Fig. 1). The concentration of DOPA in the brain at any time during the first 48 hr after MO-911 administration is not significantly higher than the control level. The epinephrine level is stable during the 48-hr period following administration of MO-911. Although epinephrine may be a substrate for MAO, adequate metabolic pathways for the metabolism of epinephrine other than that through MAO may be utilized.

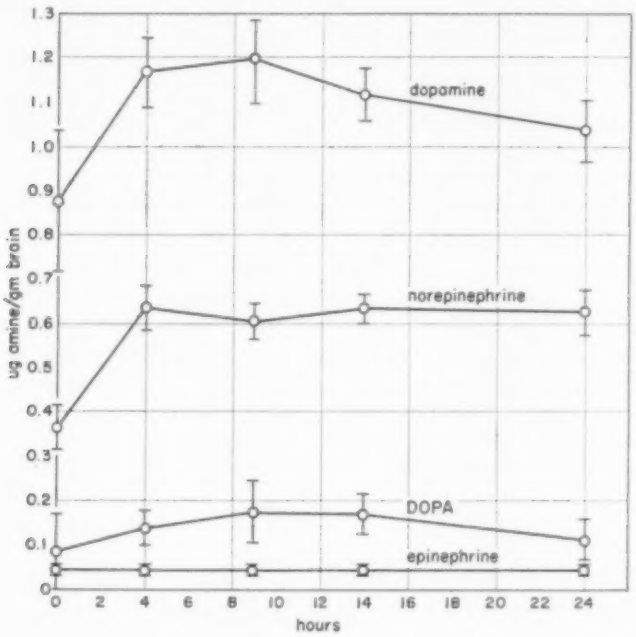


FIG. 1. Concentrations (together with their standard deviations) of DOPA, dopamine, norepinephrine and epinephrine in mouse brain following 100 mg of MO-911 (as free base) per kg intraperitoneally.

The duration of the increases in the levels of DA, NE and serotonin after MO-911, shown in Fig. 1 and Table 2, indicate that the amines return to control levels at different rates. Levels of dopamine in the brain reach a peak at about 9 hr (37 per cent above control) and decrease to control values by 48 hr. Norepinephrine concentrations in the brain are essentially stable in the period from 4 to 24 hr after MO-911 administration at a level about 72 per cent above control, and by 48 hr have decreased only slightly toward control values. This difference may be due to the relative rates of oxidative deamination of DA and NE by residual MAO activity, reported *in vitro* with a purified enzyme preparation[11] to be 100 for dopamine and 12 for norepinephrine. If these relative rates were operative *in vivo*, dopamine would fall to control levels before norepinephrine. Similarly relative rates of O-methylation could account for the result, but such rates with a purified O-methyltransferase system have been

reported by Axelrod and Tomchick[12] as essentially equal. The level of serotonin in the brain 24 and 48 hr after MO-911 remains about 40 per cent above control levels.

After administration of L-DOPA the percentage increase above control amine levels at 30 min is greatest for DOPA, and decreases in the order of metabolic conversion—DOPA, DA, NE and EP. Prior administration of the MAO-inhibitor MO-911 causes the percentage increases of DOPA, DA and NE to be much greater than after administration of L-DOPA alone, but the percentage increase is still greatest for DOPA and decreases in the order DOPA, DA, NE and EP. The failure of the epinephrine concentration to increase 30 min after the administration of L-DOPA, or of MO-911 and L-DOPA, may be due to the series of metabolic transformations prior to epinephrine formation. This is reflected in Table 2, in which it is shown that 30 min after the administration of L-DOPA, DOPA has increased over its control level by a factor of 9·4 and dopamine by a factor of 3·2, while norepinephrine is only 1·3 times its control level. These ratios after MO-911 and L-DOPA are DOPA 55, DA 11, and NE 2·2.

Increased levels of DOPA, dopamine, norepinephrine and serotonin in brain were present at the time of sacrifice of the mice exhibiting marked hyperactivity. The contribution of the increase in any one of these amines to the hyperactivity of the mice could not be determined. The slight increase in motor activity observed after administration of L-DOPA alone, however, occurred when only DOPA and dopamine levels were increased above those observed in animals showing no behavioral change.

In contrast to the changes in amines following L-DOPA, the effect of MO-911 is to elevate the levels of norepinephrine and serotonin for longer than 48 hr, while dopamine levels increase less and return to normal within 48 hr. This suggests that any effects associated with the long duration of monoamine oxidase inhibition produced *in vivo* by MO-911[1] may not be due to dopamine, but rather to norepinephrine or serotonin.

REFERENCES

1. J. D. TAYLOR, A. A. WYKES, Y. C. GLADISH and W. B. MARTIN, *Nature, Lond.* **187**, 941 (1960).
2. J. D. TAYLOR, A. A. WYKES, Y. C. GLADISH, W. B. MARTIN and G. M. EVERETT, *Fed. Proc.* **19**, 278 (1960).
3. G. M. EVERETT, *Psychopharmacology—Proceedings of the Second Meeting of the Inter-National Collegium of Neuro-Psychopharmacology, Basle, July* 1960. In press.
4. G. M. EVERETT, J. C. DAVIN and J. E. P. TOMAN, *Fed. Proc.* **18**, 613 (1959).
5. A. BERTLER and E. ROSENGREN, *Experientia* **15**, 382 (1959).
6. A. CARLSSON, M. LINDQVIST and T. MAGNUSSON, *J. Soc. Cien. Med. de Lisboa* **123**, 96 (1959).
7. P. A. SHORE and J. S. OLIN, *J. Pharmacol.* **122**, 295 (1958).
8. A. BERTLER, A. CARLSSON and E. ROSENGREN, *Acta Physiol. Scand.* **44**, 273 (1958).
9. A. CARLSSON and B. WALDECK, *Acta Physiol. Scand.* **44**, 293 (1958).
10. S. UDENFRIEND, H. WEISSBACH and D. F. BOGDANSKI, *Science* **122**, 972 (1955).
11. L. C. LEEPER, H. WEISSBACH and S. UDENFRIEND, *Arch. Biochem. Biophys.* **77**, 417 (1958).
12. J. AXELROD and R. TOMCHICK, *J. Biol. Chem.* **233**, 702 (1958).

Biochemical Pharmacology, 1961, Vol. 7, pp. 187–194. Pergamon Press Ltd., Printed in Great Britain.

EFFECTS OF PURINE ANTAGONISTS ON THE METABOLISM OF OTHER PURINE ANTAGONISTS

J. Frank Henderson* and Irene G. Junga

Department of Biological Sciences,
Stanford Research Institute, Menlo Park, California

(*Received* 5 *January* 1961)

Abstract—The effects of azaserine, thioguanine and 6-mercaptopurine on the metabolism of 4-aminopyrazolopyrimidine-^{14}C and of 4-aminopyrazolopyrimidine on the metabolism of thioguanine-^{14}C and of 6-mercaptopurine-^{14}C have been studied in Ehrlich ascites carcinoma and ascites sarcoma-180. Azaserine, thioguanine and 6-mercaptopurine not only enhanced the inhibition of tumor growth by 4-aminopyrazolopyrimidine, but also increased the amount of incorporation of the analog into tumor polynucleotides. Enhancement of the size of the pool of 4-aminopyrazolopyrimidine mononucloetides was evident during dual treatment with azaserine and thioguanine. The amount of incorporation of radioactivity from labeled 6-mercaptopurine into polynucleotides was decreased by 4-aminopyrazolopyrimidine, while little change in the size of the labeled mononucleotide pool was evident. The therapeutic effectiveness of thioguanine was increased by the co-administration of 4-aminopyrazolopyrimidine and the amount of incorporation of thioguanine into nucleic acids was somewhat increased by 4-aminopyrazolopyrimidine. These results are discussed with respect to present hypotheses concerning the anabolic conversion of purine analogs into their active derivatives.

Potentiation of carcinostatic activity has been observed when several different purine analogs have been used in combination. The pairs, 4-aminopyrazolopyrimidine (APP) plus 6-mercaptopurine (6-MP) and APP plus thioguanine (TG), show potentiation in Ehrlich ascites carcinoma (Ehrlich) and in ascites sarcoma-180 (S-180), even though APP alone is not active in sarcoma-180.[1] Combination therapy of Ehrlich carcinoma with TG plus 6-MP also shows potentiation.[2] In addition, combinations of azaserine with APP,[1] TG,[5, 6] 6-chloropurine,[5, 7] 8-azaguanine,[3] and 6-MP[3, 8, 9] have also been shown to potentiate carcinostasis in a variety of tumors. Other reports of combination chemotherapy of tumors have been reviewed by Goldin and Mantel[4].

The mechanism of action of none of these purine antimetabolites is currently known with certainty. While the primary site of azaserine action in mammalian tissues is accepted as inhibition of the biosynthesis of purines *de novo*,[10] there remain secondary aspects of the action of this drug which are unclear.[11–13] Thus, it is difficult if not impossible to ascertain exactly the mechanisms by which combinations of these agents potentiate carcinostasis. However, this phenomenon offers contributory evidence that the drugs used in combination have different mechanisms of action, for otherwise only additive effects would be expected.

One possible factor in potentiation which can readily be investigated is the effect of one drug on the metabolism of the other. Because the active forms of many of the

* Present address: Department of Pharmacology, The George Washington University School of Medicine, Washington, D.C., U.S.A.

purine antimetabolites are believed to be their ribonucleotides,[14-16] it seemed useful to look for alterations in the concentrations of the ribonucleotides of the drugs in the umor cells during potentiation. In two cases of potentiation with azaserine changes of this sort have been shown to occur. LePage[12] found that a TG-resistant subline of the Ehrlich carcinoma could be restored to sensitivity in a potentiative manner when treated with both TG and azaserine, and that the concentration of the TG-ribonucleotide and the incorporation of TG into nucleic acids increased concomitantly. Paterson[13] also observed increased formation of 6-MP ribonucleotide following co-administration of azaserine. However, studies of the simultaneous metabolism of two purine analogs, when used in combination therapy, have not been reported previously.

Biochemical and biological studies of the action and the metabolism of the adenine analog, APP, have recently been made in this laboratory.[1, 15] Combinations of this compound with 6-MP, TG or azaserine potentiated carcinostasis. Therefore it was of interest to determine the effect of these drugs on the metabolism of APP in two mouse tumors, one sensitive to the antimetabolite when used alone (Ehrlich ascites carcinoma), the other sensitive only in combination therapy (ascites sarcoma-180). To complete the study, the influence of APP on the metabolism of 6-MP and of TG in the same tumors was also studied.

METHODS

4-Aminopyrazolopyrimidine-6-^{14}C was a gift of Dr. L. L. Bennett, Jr., Southern Research Institute, Birmingham, Ala., to whom we are very grateful. The compound was subjected to further purification in this laboratory and when used contained less than 1 per cent impurity. As measured, this material had a specific activity of 2000 counts/min per μg. 6-Mercaptopurine-8-^{14}C was purchased from Isotope Specialties Co., Inc., and diluted with non-radioactive material until its specific activity was 475 counts/min per μg. Thioguanine-8-^{14}C was synthesized by Dr. G. A. LePage of this laboratory. His gift of this material, which had a specific activity of 2600 counts/min per μg, is greatly appreciated. Azaserine and non-radioactive APP were obtained from the Cancer Chemotherapy National Service Center, National Cancer Institute; 6-MP from the California Corporation for Biochemical Research; and the TG was synthesized in this laboratory. All compounds were dissolved in 0·154 M sodium chloride before use.

Female Swiss mice, 25–30 g, were inoculated with 1×10^6 Ehrlich ascites carcinoma cells or 6×10^6 sarcoma-180 ascites cells, and used 6 days thereafter.

Ascites tumor cells were removed from the mice by capillary pipette after laporotomy, separated from the ascitic fluid by centrifugation, and extracted three times with cold 0·2 M perchloric acid. The combined extracts were neutralized with potassium hydroxide, chilled and the potassium perchlorate was removed. The acid-insoluble residue was suspended in 0·4 M perchloric acid and heated at 100 °C for 30 min. This hot extract was combined with one 0·4 M perchloric acid-washing of the precipitate, neutralized with potassium hydroxide, the potassium perchlorate was removed in the cold, and the solution was evaporated to dryness. Each sample was redissolved in 1 ml of 0·05 M hydrochloric acid and an aliquot was taken for radioactivity measurements in a micro-thin window gas-flow counter. The radioactivity in this fraction was used as a measure of incorporation of the analogs into the combined nucleic acids.

Metabolites of APP-^{14}C were separated from the neutralized cold acid extracts by chromatography on 10×40 mm Dowex-1-formate columns. The effluent, plus 10 ml

Vol. 7
1961

of water, contained the free base and ribonucleoside, which were not separated because it had previously been found that the ribonucleoside usually comprised only about 10 per cent of this fraction.[15] To the column were added 15 ml of 0·05 M formic acid to elute the degradation products of APP, which consisted of 4-hydroxypyrazolopyrimidine plus several unidentified compounds;[15] 15 ml of 2·0 M formic acid to elute the ribonucleoside monophosphate of this drug; 15 ml of 4·0 M formic acid containing 0·2 M ammonium formate to elute the ribonucleoside diphosphate; and 30 ml of 2·0 M formic acid containing 2·5 M ammonium formate to elute the ribonucleoside triphosphate. Further details of the separation and identification of these metabolites have been presented previously.[15]

Metabolites of TG-^{14}C and of 6-MP-^{14}C were separated by a manual chromatographic procedure based on the gradient elution chromatographic separations of these compounds by Moore and LePage[16] and by Paterson[13]. Neutralized tissue extracts were poured on to 10 × 40 mm Dowex-1-formate columns. The effluent, plus 15 ml of 0·05 M formic acid, contained the free bases and ribonucleosides of these compounds, which were not further separated. Fifteen milliliters of 3·0 M formic acid eluted the degradation products, 6-thio-uric acid and 6-thio-xanthine, which were not further separated. Thirty milliliters of 2·0 M formic acid containing 2·5 M ammonium formate eluted the ribonucleotides of both TG and 6-MP.

Each fraction was evaporated to dryness and ammonium formate, if present, was sublimed off. Each sample was redissolved in 1 ml of 0·05 N hydrochloric acid and an aliquot was taken for radioactivity measurements.

RESULTS

To determine the effects of one drug on the metabolism of the other, both drugs of the pair under consideration were simultaneously injected intraperitoneally into mice bearing 6-day tumors. One of each drug pair was labeled with radiocarbon. The following combinations were employed: APP-^{14}C plus azaserine; APP-^{14}C plus TG; APP-^{14}C plus 6-MP; TG-^{14}C plus APP; and 6-MP-^{14}C plus APP. The doses used in all cases were those employed in therapy: 750 μg of 6-MP; 200 μg of APP; 25 μg of TG; and 5 μg of azaserine.[1] One, two and four hours later the tumor cells were removed from these animals, extracted with perchloric acid, and the concentrations of radioactive drug metabolites determined as described above. All results are expressed as the averages of determinations from three to six mice analyzed separately, and the variation among samples averaged 10 per cent. Radioactivity is presented as microgram-equivalents of the injected free base per gram wet weight of tissue.

Table 1 shows the effect of azaserine on the metabolism of APP-^{14}C. The principal effect of azaserine was to increase the amount of ribonucleotides of APP found in these cells. This increase was greater in sarcoma-180 than in Ehrlich carcinoma, a difference which can be correlated with therapeutic response. The former tumor is not sensitive to APP alone, but becomes quite sensitive to combination treatment.[1] There was also an increase in the amount of incorporation of APP-^{14}C into the nucleic acid fraction at the early time periods. (This incorporation has previously been shown to be by true internucleotide linkage.[15])

The effect of TG on the metabolism of APP-^{14}C, as shown in Table 2, was somewhat similar to that exerted by azaserine. The amount of ribonucleotides of APP was consistently increased in Ehrlich carcinoma after TG co-administration. In sarcoma-180

TABLE 1. EFFECT OF AZASERINE ON THE METABOLISM OF APP-^{14}C

Tumor	Conditions*	Drug metabolites (μg base per g of tissue)											
		Nucleic acid incorporation			Nucleotides			Base plus nucleoside			Degradation products		
		1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr
Ehrlich	control	1·87	1·78	1·12	26·9	23·8	18·7	0·204	0·242	0·097	1·96	1·73	0·596
	+ azaserine	3·50	1·91	1·23	31·5	29·2	20·1	0·515	0·150	0·144	1·96	1·55	0·382
S-180	control	1·32	1·08	0·994	21·2	20·6	20·0	0·204	0·149	0·161	0·997	0·911	0·661
	+ azaserine	1·86	1·52	0·981	32·1	27·8	22·3	0·246	0·114	0·086	1·54	1·43	0·554

* Tumor-bearing mice were each injected with 200 μg of APP-^{14}C and 5 μg of azaserine.

TABLE 2. EFFECT OF TG ON THE METABOLISM OF APP-^{14}C

Tumor	Conditions*	Drug metabolites (μg base per g of tissue)											
		Nucleic acid incorporation			Nucleotides			Base plus nucleoside			Degradation products		
		1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr
Ehrlich	control	1·87	1·78	1·12	26·9	23·8	18·7	0·204	0·242	0·097	1·96	1·73	0·596
	+ TG	3·49	3·83	2·38	31·8	26·2	22·6	0·404	0·538	1·69	1·77	1·29	0·671
S-180	control	1·32	1·08	0·994	21·2	20·6	20·0	0·204	0·149	0·161	0·977	0·911	0·661
	+ TG	2·91	1·37	0·663	36·4	19·1	15·8	0·528	0·399	0·112	2·01	0·746	0·687

* Tumor-bearing mice were each injected with 200 μg of APP-^{14}C and 25 μg of TG.

TABLE 3. EFFECT OF 6-MP ON THE METABOLISM OF APP-^{14}C

Tumor	Conditions*	Drug metabolites (μg base per g of tissue)											
		Nucleic acid incorporation			Nucleotides			Base plus nucleoside			Degradation products		
		1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr
Ehrlich	control	1·87	1·78	1·12	26·9	23·8	18·7	0·204	0·242	0·097	1·96	1·73	0·596
	+ MP	4·99	2·00	1·51	25·8	20·8	17·1	0·320	0·183	0·100	1·26	1·18	0·444
S-180	control	1·32	1·08	0·994	21·2	20·6	20·0	0·204	0·149	0·161	0·977	0·911	0·661
	+ MP	4·02	2·75	1·70	21·9	17·9	13·1	0·211	0·290	0·559	1·40	1·15	0·631

* Tumor-bearing mice each received 200 μg of APP-^{14}C and 750 μg of 6-MP.

TABLE 4. EFFECT OF APP ON THE METABOLISM OF TG-^{14}C

Tumor	Conditions*	Drug metabolites (μg base per g of tissue)											
		Nucleic acid incorporation			Nucleotides			Base plus nucleoside			Degradation products		
		1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr
Ehrlich	control	2·64	0·907	0·632	4·09	2·63	1·75	3·77	1·32	1·10	3·32	1·18	0·835
	+ APP	3·16	1·58	0·892	3·59	2·42	1·92	2·43	0·809	0·602	3·42	1·08	0·906
S-180	control	1·01	0·900	1·18	2·89	2·22	1·88	5·45	2·36	2·80	1·92	1·93	1·98
	+ APP	5·88	2·40	1·16	2·78	2·28	2·00	1·83	1·49	1·10	1·19	1·36	1·32

* Tumor-bearing mice each received 25 μg of TG-^{14}C and 200 μg of APP.

TABLE 5. EFFECT OF APP ON THE METABOLISM OF 6-MP-^{14}C

Tumor	Conditions*	Drug metabolites (μg base per g of tissue)											
		Nucleic acid incorporation			Nucleotides			Base plus nucleoside			Degradation products		
		1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr
Ehrlich	control	1·15	1·09	0·940	4·92	4·86	2·94	0·232	0·363	0·200	1·34	1·84	1·62
	+ APP	0·900	0·717	0·950	4·62	3·80	2·28	0·224	0·303	0·220	1·10	1·41	1·35
S-180	control	0·565	0·758	0·364	5·07	5·16	2·20	0·237	0·297	0·220	1·45	2·18	1·59
	+ APP	0·520	0·336	0·439	4·71	2·77	2·53	0·225	0·310	0·159	2·04	1·03	1·07

* Tumor-bearing mice were each injected with 750 μg of 6-MP-^{14}C and 200 μg of APP.

increased ribonucleotide levels were noted at the first hour, but in this tumor the concentration of the ribonucleotide of the analog decreased more quickly than in the controls, until it was significantly less than control values at 4 hr. Increased amounts of incorporation into nucleic acids were also evident in Ehrlich carcinoma throughout the time course studied, and in sarcoma-180 at the first hour.

Table 3 shows the effect of 6-MP on the metabolism of APP-^{14}C, which differed from that of azaserine or TG. While the amount of incorporation of the radioactive drug into the nucleic acids was increased by the co-administration of 6-MP, the acid-soluble ribonucleotide concentration in all cases was equal to or less than the control values. Other fractions were only slightly changed.

To complete this study, the metabolism of the two other purines involved both in potentiation of carcinostasis and alterations in the metabolism of APP was investigated. Table 4 shows the effect of APP on the metabolism of TG-^{14}C in these tumors. More TG was incorporated into nucleic acids and less into acid-soluble ribonucleotides following the co-administration of APP than in the control cells. While little or no change in the amount of degradation products was observed, the content of free base of TG was lowered after combination therapy. In this case it appears that the total amount of TG present in the APP-treated cells was less than in the controls. (It might be pointed out that very little desulfuration of TG occurs in these tumors,[11] so radioactivity can be equated to drug content with considerable reliability.)

Finally, the effect of APP on the metabolism of 6-MP-^{14}C was studied (Table 5). The amount of incorporation of radioactivity from 6-MP-^{14}C into the tumor nucleic acids was decreased in some cases by the co-administration of APP, while the concentrations of acid-soluble ribonucleotides of 6-MP were slightly below the control values. (While it has been shown that most of the radioactivity in these tumors really is associated with 6-MP and its metabolites,[13] some desulfuration does occur. It is particularly likely to be the case that much of the radioactivity in the nucleic acid fraction was contained in adenine and guanine.[17, 18])

DISCUSSION

It is evident from the results presented that two purine antimetabolites may be metabolized differently in the presence of each other than each is by itself, and that different tumors vary in the extent to which this happens. In the cases observed, however, the alterations have all been quantitative, rather than qualitative, and on the whole have not been large. The most notable and consistent changes were in two fractions: the concentration of acid-soluble ribonucleotides of the drugs (their presumptive active forms), and the amount of ribonucleotide incorporated into the nucleic acids. Increases were noted in the amounts incorporated into nucleic acid, while both increases and decreases were found in acid-soluble ribonucleotide concentrations. Thus, the concentrations of the ribonucleotides of the potentiating drugs did not always parallel their increased carcinostatic activity. This appears to be an exception to the commonly accepted concept which relates the amount of ribonucleotide formation by purine analogs to the extent of tumor inhibition.[14–16]

Two general mechanisms may be adduced to explain such phenomena. The two drugs and their metabolites may compete for enzymes and co-factors involved in their metabolism; or, more likely, these changes in metabolism may be the direct results of the actions of both drugs on the purine metabolism of the cells, each drug probably having

a different action. For example, analogs may compete either for pyrophosphorylases involved in their conversion to nucleotides, or for degradative enzymes. Also, a drug which may affect one area of purine metabolism may indirectly make its companion drug more or less susceptible to the action of the enzymes concerned. The thirty-fold range in the doses of the purine analogs used also may influence the effects observed.

Azaserine is a special case. It is an irreversible inhibitor the action of which depends only on those molecules which attach to the proper enzyme.[10, 19] This happens within minutes after its injection and it seems improbable that the purine analogs interfere with this process. Whatever effect the purines might have on excess azaserine molecules, it seems unlikely that the carcinostatic process would be affected. Since azaserine at the dose used affects purine metabolism directly only by interruption of the *de novo*-biosynthesis of purines,[20] it is suggested that its effects on the metabolism of APP (and that of TG and 6-MP as well[12, 13]) are caused by perturbations in the metabolism of purine ribonucleotides resulting from inhibition of *de novo*-biosynthesis, or by some effect of the formylglycineamide ribonucleotide which accumulates during treatment with azaserine.

Thus, the mechanisms by which these alterations in drug metabolism come about remain unclear, as are the processes by which these drug combinations potentiate carcinostasis. One factor probably is the increase in the amount of the ribonucleotides of the analogs which is present in the cells after treatment with two drugs, as compared to that after only one, but, as pointed out above, this is not a complete explanation. It is also conceivable that the disruptive action of one drug may make certain aspects of purine metabolism unusually sensitive to inhibition by the other analog.

Acknowledgements—This work was supported by Contract no. SA-43-ph-3068 with the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, and by grants from the U.S. Public Health Service (CY-4551) and the Gustavus and Louise Pfeiffer Research Foundation.

The authors wish to express their appreciation to Dr. G. A. LePage, in whose laboratory this work was done, and to acknowledge the assistance of Miss Noel Howard in making the radioactivity measurements.

REFERENCES

1. J. F. Henderson and I. G. Junga, *Cancer Res.* **20**, 1618 (1960).
2. J. F. Henderson and I. G. Junga, *Biochem. Pharmacol.* **5**, 167 (1960).
3. H. E. Skipper, *Ann. N.Y. Acad. Sci.* **60**, 267 (1954).
4. A. Goldin and N. Mantel, *Cancer Res.* **17**, 635 (1957).
5. G. S. Tarnowski and C. C. Stock, *Cancer Res.* **17**, 1033 (1957).
6. A. C. Sartorelli and G. A. LePage, *Cancer Res.* **18**, 938 (1958).
7. A. C. Sartorelli and B. A. Booth, *Cancer Res.* **20**, 198 (1960).
8. J. J. Jaffe and H. G. Mautner, *Cancer Res.* **20**, 381 (1960).
9. C. C. Stock, H. C. Reilly, S. M. Buckley, D. A. Clarke and C. P. Rhoades, *Acta Unio Int. Contra Cancrum* **11**, 186 (1955).
10. S. C. Hartman, B. Levenberg and J. M. Buchanan, *J. Amer. Chem. Soc.* **77**, 501 (1955).
11. A. C. Sartorelli, G. A. LePage and E. C. Moore, *Cancer Res.* **18**, 1232 (1958).
12. G. A. LePage, *Cancer Res.* **20**, 403 (1960).
13. A. R. P. Paterson, *Canad. J. Biochem. Physiol.* **37**, 1011 (1959).
14. R. W. Brockman, L. L. Bennett, Jr., M. S. Simpson, A. R. Wilson, J. R. Thomson and H. E. Skipper, *Cancer Res.* **19**, 659 (1959).
15. J. F. Henderson and I. G. Junga, *Cancer Res.* **21**, 118 (1961).
16. E. C. Moore and G. A. LePage, *Cancer Res.* **18**, 1075 (1958).
17. G. B. Elion, S. Bieber and G. H. Hitchings, *Ann. N.Y. Acad. Sci.* **60**, 297 (1954).
18. N. H. Carey and H. G. Mandel, *Biochem. Pharmacol.* **5**, 64 (1960).
19. J. Greenlees and G. A. LePage, *Cancer Res.* **16**, 808 (1956).
20. G. A. LePage and A. C. Sartorelli, *Texas Rep. Biol. Med.* **15**, 109 (1957).

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 195–220. Pergamon Press Ltd., Printed in Great Britain.

VERSUCHE ZUR ENTDECKUNG NEUER FUNGISTATIKA—III

8-HYDROXY-CHINOLIN-DERIVATE, NITROSO-VERBINDUNGEN UND OXYME

TIBOR ZSOLNAI

Mitteilung aus dem Hygienischen Institut* der Medizinischen Universität in Debrecen, Ungarn

(*Received* 24 *March* 1961)

Abstract—The fungistatic effect of forty one 8-hydroxychinolin derivatives, thirty three nitroso compounds, twenty oxymes and ten phenanthrolin derivatives have been investigated on liquid mash-medium containing 10% cattle serum, and on solid medium containing 3% agar. At the same time the mechanism of action was studied using those compounds which proved to be most active. A relationship was sought between the chelate-complex-forming ability and the fungistatic efficiency of the compounds. The effect of the agents on the fungistatic activity was investigated in relation to reaction mechanism.

The 8-hydroxy-chinolin derivatives, which were found to be most effective, exercise their fungistatic action by interfering with indirect intercellular oxidation of SH-groups in important sulphhydryl enzymes. The same conclusion has been reached by Rubbo and his collaborators using bacteria.

The mechanism of action of 2:2′-dipyridyl and 1:10-phenanthrolin-hydrochloride proved to be entirely different from those of 8-hydroxy-chinolin-derivatives. These compounds exercise their fungistatic activity simply by means of intercellular chelation of the heavy metal components in certain enzymes of fungus-cells. These enzymes are inactivated and this prevents multiplication.

No mechanism for the fungistatic action of nitroso-compounds could be proposed.

IM ERSTEN Teil unserer Mitteilungsserie befassten wir uns mit der *in vitro* ausgeübten fungistatischen Wirkung von Phenol-Derivaten,[1] im II Teil aber mit derjenigen verschiedener Nitro-Verbindungen, sowie mit dem Wirkungsmechanismus derer unter diesen, die dabei aktiv gefunden wurden.[2]

In dieser Mitteilung wollen wir uns mit der fungistatischen Wirkung der 8-Hydroxy-chinolin-Derivate, der Nitroso-Verbindungen und der Oxyme beschäftigen und unsere Versuche zur Erkennung des Wirkungsmechanismus derjenigen bekannt machen, die unter ihnen am intensivsten wirksam gefunden wurden.

Die 8-Hydroxy-chinolin-Derivate bilden eine eigentümliche Gruppe der Fungistatika phenolischer Natur. Nur jene Mitglieder dieser Familie von Verbindungen haben eine fungistatische Wirkung, in denen die phenolische Hydroxy-Gruppe frei ist und an das Carbon-Atom 8 des Chinolin-Skeletts gebunden wird; die 5- oder 6-Hydroxy-chinolin-Derivate bleiben dagegen wirkungslos. Unter den 8-Hydroxy-chinolin-Derivaten wurde vor allem der "Mutterstoff" selbst, d.h. das 8-Hydroxy-chinolin[3–10] auf die verschiedenen Pilzstämme *in vitro* als beträchtlich fungistatisch bzw. fungizid wirksam gefunden, sowie dessen Kupfer-Chelat-Komplex,[6–11] ferner das 8-Hydroxy-

* Vorstand: Universitätsprofessor Dr. med. Endre Jeney.

chinaldin[3, 4] und die 5:7-Dihalogen-Derivate des 8-Hydroxy-chinolins und des 8-Hydroxy-chinaldins und deren Kupfer-Chelate-Komplexe.[3, 4, 9–17]

Das 8-Hydroxy-chinolin und das 5:7-Dichlor-8-hydroxy-chinaldin erwiesen sich in den Fällen von Fussmykosen auch klinisch als therapeutisch wirksam.[12, 18, 19]

Die 8-Hydroxy-chinolin-Derivate sind *keine spezifischen Antimikotyka*. Ihre entwicklungshemmende Wirkung beschränkt sich keineswegs auf die verschiedenen Pilzarten, sondern erstreckt sich auf alle gram-positiven und zahlreiche gram-negativen Bakterienstämme,[20–28] auf Tuberkulose-Bakterien,[4, 29–33] ausserdem auch auf einzelne Protozoons (z.B. auf *Entamoeba histolytica*[34–44] und auf *Trichomonas vaginalis*[36, 45, 46]).

Das 8-Hydroxy-chinolin und seine Derivate haben eine stark Chelate-Komplex-bildende Eigenschaft. Unter ihrer bakteriostatischen und fungistatischen Wirkung sowie ihrer Chelate-Komplex-bildenden Eigenschaft wurde das Bestehen eines Zusammenhanges durch zahlreiche Autoren nachgewiesen.[5, 21–32, 47]

Aber nicht nur die 8-Hydroxy-chinolin-Derivate verfügen über eine hochgradige Chelate-Komplex-bildende Eigenschaft und zugleich über eine bakteriostatische und fungistatische Wirkung, sondern—unter anderen mehr—auch verschiedene *o*-Nitroso-phenol-Derivate,[4, 25, 26, 28] Hydroxam-Säuren[48, 49] und die *o*-Phenanthroline.[50, 51]

Wir hielten für zweckmässig, unsere Ergebnisse, die wir an Hand der Untersuchung der fungistatischen Wirkung der Nitroso-phenole und Phenanthroline, sowie durch das Studium deren Wirkungsmechanismus erreichten, zugleich mit der Wirkung der 8-Hydroxy-chinolin-Derivate mitzuteilen, da es für wahrscheinlich zu erachten ist, dass diejenigen unter ihnen, welche mit Schwermetall-Ionen Chelate-Komplexe zu bilden vermögen—auch trotz der abweichenden chemischen Struktur—ihre fungistatische Wirkung auf Grund eines mehr oder weniger ähnlichen biochemischen Mechanismus ausüben wie das 8-Hydroxy-chinolin selbst und dessen aktive Derivate.

Wir hielten es für begründet, unsere Untersuchungen ausserdem auch auf die Chelate-Komplex-Bildung unfähigen Nitroso-Verbindungen und auf ihre formalen Tautomere, d.h. die Oxyme zu erstrecken, damit man an Hand der Vergleichung der Untersuchung dieser mit den Resultaten, die durch die Untersuchung der zur Chelate-Komplex-Bildung geneigten Nitroso-Verbindungen erhalten wurden, einerseits Zusammenhänge zwischen der chemischen Struktur und der fungistatischen Wirkung der Nitroso-Verbindungen finden möge; andererseits aber deshalb, damit wir über die Abhängigkeit ihrer fungistatischen Wirkung von ihrer Chelate-Komplex-bildenden Fähigkeit mit einem weiteren Geltungsbereich Feststellungen machen können.

Die Mehrheit der untersuchten Verbindungen haben wir mittels der in der chemischen Fachliteratur bekannten Methoden oder deren Anwendung auf Analoga selbst synthetisiert; die Minderheit stand uns in der Form von Handelsprodukten in "pro analysi" Qualität zur Verfügung.

Die im Laufe der Untersuchung der fungistatischen Wirkung dieser Verbindungen verfolgten Methoden waren mit denen, die in dem ersten Teil unserer Mitteilungsserie bereits beschrieben wurden[1] ganz übereinstimmend, deshalb wollen wir hier darauf nicht mehr ausführlich eingehen.

Es soll diesmal nur erwähnt werden, dass wir auch zu diesen Untersuchungen—wie auch für unsere früheren Forschungen bezüglich der fungistatischen Wirkung der Phenol-Derivate[1] und Nitro-Verbindungen[2]—flüssigen Maische-Nährboden mit 10% Rinderserum-Gehalt sowie serumfreien festen Maische-Agar-Nährboden verwendet haben. Das pH der Kulturmedien betrug 6,0–6,2.

Vol. 1961

ERGEBNISSE UND BESPRECHUNG

Die Ergebnisse, die wir im Laufe der Untersuchung der *in vitro* ausgeübten fungistatischen Wirkung der 8-Hydroxy-chinolin-Derivate, Nitroso-Verbindungen, Oxyme und der zum Vergleich untersuchten Phenanthrolin-Derivate erhielten, fassten wir in den Tabellen 1, 2, und 3 zusammen.

TABELLE 1. DIE FUNGISTATISCHE WIRKUNG VON 8-HYDROXY-CHINOLIN-DERIVATEN, NITROSO-VERBINDUNGEN UND OXYMEN AUF FLÜSSIGEM SERUM-MAISCHE NÄHRBODEN

Nr.	Verbindungen	*Penicillium simplicissimum*	*Aspergillus niger*	*Trichothecium roseum*	*Candida albicans*	*Achorion quinckeanum*	*Trichophyton gypseum*	*Epidermophyton Kaufman-Wolff*
	I. *8-Hydroxy-chinolin-Derivate*							
F 445	8-Hydroxy-chinolin	250 000	250 000	250 000	250 000	250 000	250 000	250 000
F 446	Kupfer-8-hydroxy-chinolinat	10 000	10 000	10 000	10 000	25 000	25 000	50 000
F 447	Zink-2-methyl-8-hydroxy-chinolinat	—	—	10 000	—	10 000	10 000	10 000
F 448	Zink-2:5-dimethyl-8-hydroxy-chinolinat	—	—	10 000	—	10 000	10 000	10 000
F 449	5:7-Dichlor-8-hydroxy-chinolin	250 000	250 000	250 000	250 000	250 000	250 000	250 000
F 450	5:7-Dibrom-8-hydroxy-chinolin	100 000	100 000	100 000	100 000	100 000	100 000	100 000
F 451	5:7-Dijod-8-hydroxy-chinolin	50 000	25 000	50 000	25 000	50 000	50 000	100 000
F 452	5-Chlor-7-jod-8-hydroxy-chinolin	250 000	250 000	250 000	250 000	250 000	250 000	250 000
F 453	2-Methyl-5:7-dibrom-8-hydroxy-chinolin	5000	5000	10 000	10 000	25 000	10 000	25 000
F 454	5:7-Dinitro-8-hydroxy-chinolin	100 000	100 000	100 000	250 000	250 000	250 000	250 000
F 455	5-Nitroso-8-hydroxy-chinolin hydrochlorid	(5000)	—	5000	5000	5000	5000	5000
F 456	7-(1'-Piperidino-methyl)-8-hydroxy-chinolin dihydrochlorid	(5000)	—	5000	(5000)	10 000	10 000	10 000
F 457	7-(α-(2'-Thiazolyl amino) benzyl)-8-hydroxy-chinolin	10 000	10 000	10 000	10 000	25 000	25 000	25 000
F 458	5-Phenyl-azo-8-hydroxy-chinolin	5000	5000	10 000	5000	25 000	25 000	25 000
F 459	5-(4'-Methyl-phenyl-azo)-8-hydroxy-chinolin	5000	(5000)	5000	5000	25 000	10 000	25 000
F 460	5-(4'-Aethoxy-phenyl-azo)-8-hydroxy-chinolin	5000	(5000)	5000	(5000)	10 000	10 000	10 000
F 461	5-(4'-Chlor-phenyl-azo)-8-hydroxy-chinolin	5000	5000	10 000	5000	25 000	25 000	25 000
F 470	5-Phenyl-azo-7-brom-8-hydroxy-chinolin	50 000	25 000	50 000	50 000	100 000	100 000	100 000
F 471	5-(4'-Methyl-phenyl-azo)-7-brom-8-hydroxy-chinolin	50 000	25 000	50 000	50 000	100 000	100 000	100 000
F 472	5 (4'-Aethoxy-phenyl-azo)-7-brom-8-hydroxy-chinolin	50 000	25 000	50 000	50 000	100 000	100 000	100 000
F 473	5-(4'-Chlor-phenyl-azo)-7-brom-8-hydroxy-chinolin	50 000	25 000	50 000	50 000	100 000	100 000	100 000
F 474	5-Phenyl-azo-7-brom-8-hydroxy-chinolin-2'-carbonsäure	50 000	25 000	50 000	50 000	100 000	100 000	100 000
F 475	5-Phenyl-azo-7-brom-8-hydroxy-chinolin-4'-carbonsäure	50 000	25 000	50 000	50 000	100 000	100 000	100 000
F 476	5-Phenyl-azo-7-brom-8-hydroxy-chinolin-4'-sulfamid	50 000	25 000	50 000	50 000	100 000	100 000	100 000
F 477	5-(4'-(3'':6''-Dimethyl-2''-pirimidyl-amino-sulfonyl)-phenyl-azo)-7-brom-8-hydroxy-chinolin	50 000	25 000	50 000	50 000	100 000	100 000	100 000
F 478	5-(4'-(6''-Methoxy-3''-piridazyl-amino-sulfonyl) phenyl-azo)-7-brom-8-hydroxy chinolin	10 000	10 000	25 000	25 000	50 000	50 000	50 000
F 479	5-(4'-(5''-Brom-2''-thiazolyl-amino-sulfonyl)-phenyl-azo)-7-brom-8-hydroxy-chinolin	10 000	10 000	10 000	10 000	25 000	25 000	25 000

TABELLE 1—*fortges.*

Nr.	Verbindungen	*Penicillium simplicissimum*	*Aspergillus niger*	*Trichothecium roseum*	*Candida albicans*	*Achorion quinckeanum*	*Trichophyton gypseum*	*Epidermophyton Kaufman-Wolff*
F/480	5-(4'-Chlor-phenyl-azo)-7-(1''-piperidinomethyl)-8-hydroxy-chinolin	50 000	25 000	50 000	25 000	50 000	50 000	100 000
F/483	Di-(8-hydroxy-5-chinolyl)-methan	—	—	—	—	(5000)	(5000)	(5000)
F/484	Zink-2:6-dimethyl-10-hydroxy-1:5-phenanthrolinat	—	—	—	5000	(5000)	(5000)	5000
F/485	Zink-2:6:9-Trimethyl-10-hydroxy-1:5-phenanthrolinat	—	—	—	5000	(5000)	(5000)	5000
	II. *N-Nitroso-amine*							
F/486	N-Nitroso-diphenylamin	—	—	5000	—	10 000	10 000	10 000
	III. *C-Nitroso-arylamine*							
F/492	4-Nitroso-dimethylanilin	100 000	50 000	50 000	10 000	250 000	250 000	250 000
F/493	4-Nitroso-diaethylanilin	50 000	25 000	50 000	25 000	250 000	250 000	250 000
F/494	4-Nitroso-diphenylamin	—	—	5000	—	10 000	10 000	10 000
F/495	1-Nitroso-2-naphtylamin	10 000	5000	10 000	5000	10 000	10 000	25 000
F/496	4-Nitroso-1-dimethylamino-naphtalin	—	—	10 000	—	10 000	5000	5000
	IV. *Nitroso-phenole und ihre Schwermetall-Komplexe*							
F/497	4-Nitroso-phenol	—	—	5000	—	5000	10 000	5000
F/498	Kupfer 2 nitroso-phenolat	—	—	5000	—	5000	5000	10 000
F/499	Kupfer-2-nitroso-4-methyl-phenolat	—	—	5000	—	10 000	10 000	10 000
F/500	Kupfer-2-nitroso-5-methyl-phenolat	—	—	—	—	10 000	10 000	10 000
F/501	Kupfer-2-nitroso-6-methyl-phenolat	—	—	—	—	10 000	10 000	10 000
F/502	Kupfer-2-nitroso-4-chlor-phenolat	—	—	—	—	5000	5000	5000
F/503	Kupfer-2-nitroso-6-chlor-phenolat	—	—	—	—	5000	5000	5000
F/504	Zink-2-nitroso-5-diaethyl-amino-phenolat	—	—	5000	—	5000	5000	5000
F/508	1-Nitroso-2-naphtol	10 000	10 000	10 000	5000	100 000	100 000	100 000
F/509	2-Nitroso-1-naphtol	10 000	10 000	10 000	5000	100 000	100 000	100 000
F/510	4-Nitroso-1-naphtol	—	—	5000	—	25 000	25 000	25 000
F/511	9-Nitroso-10-phenanthrol	10 000	10 000	25 000	10 000	100 000	100 000	100 000
	V. *Sonstige Nitroso—Verbindungen*							
F/517	3:5-Dimethyl-4-nitroso-pyrazol	50 000	10 000	10 000	25 000	25 000	25 000	25 000
F/518	1-Phenyl-3:5-dimethyl-4-nitroso-pyrazol	250 000	250 000	100 000	250 000	50 000	50 000	50 000
	VI. *Oxyme*							
F/522	2-Hydroxy-benzaldoxym	5000	(5000)	5000	—	5000	5000	5000
F/536	1:2-Naphtochinon-dioxym	10 000	5000	10 000	10 000	25 000	25 000	25 000
F/537	1:2-Naphtochinon-1-oxym-2-amino-guanidon	—	—	10 000	—	10 000	10 000	10 000
	VII. *Zum Vergleich untersuchte Verbindungen*							
F/539	2:2'-Dipiridyl	25 000	25 000	25 000	25 000	25 000	25 000	25 000
F/540	1:10-Phenanthrolin hydrochlorid	50 000	50 000	50 000	50 000	50 000	50 000	100 000

Die in der Tabelle stehenden Ziffern bezeichnen die Reziprokwerte jener Verdünnung der einzelnen Verbindungen, welche zur vollkommenen Entwicklungshemmung führen.
— = Die untersuchte Verbindung übt auch in der angewandten niedrigsten Verdünnung—in 1:5000—keine fungistatische Wirkung aus.
(5000) = Die untersuchte Verbindung kann auch in der angewandten niedrigsten Verdünnung—in 1:5000—nur eine teilweise Entwicklungshemmung des Pilzstammes herbeiführen.

Bei der Übersicht der Ergebnisse der Tabellen 1 und 2 sieht man, dass das Hydroxy-Radikal in einem jeden der von uns untersuchten Derivate des 8-Hydroxy-chinolins unsubstituiert bleibt, so ist jeder von ihnen zur Bildung der Metall-Chelate-Komplexe fähig. Die Mehrheit dieser hat sich auf flüssigen Nährboden fungistatisch mehr oder

Vol. 7
1961

TABELLE 2. 8-HYDROXY-CHINOLIN-DERIVATE, NITROSO-VERBINDUNGEN UND OXYME, WELCHE IN FLÜSSIGEM SERUM-MAISCHE NÄHRBODEN AUF JEDE UNTERSUCHTE PILZSTÄMME VOLLSTÄNDIG UNWIRKSAM SIND

F/462	5-Phenyl-azo-8-hydroxy-chinolin-2′-carbonsäure
F/463	5-Phenyl-azo-8-hydroxy-chinolin-4′-carbonsäure
F/464	5-Phenyl-azo-8-hydroxy-chinolin-4′-sulfamid
F/465	5-(4′-(3″:6″-Dimethyl-2″-pirimidyl-amino-sulfonyl)-phenyl-azo)-8-hydroxy-chinolin
F/466	5-(4′-(6″-Methoxy-3″-piridazyl-amino-sulfonyl)-phenyl-azo)-8-hydroxy-chinolin
F/467	5-(4′-(2″-Thiazolyl-amino-sulfonyl)-phenyl-azo)-8-hydroxy-chinolin
F/468	5-(1′-Naphtyl-azo)-8-hydroxy-chinolin
F/469	4:4′-Di-(8″-Hydroxy-5″-chinolyl-azo)-diphenyl
F/481	N-(8′-Hydroxy-5′-chinolyl)-1:4-benzochinon-monoimin
F/482	N-(8′-Hydroxy-5′-chinolyl)-1:4-benzochinon-diimin
F/487	N-Nitroso-diaethylamin
F/488	N-Nitroso-di-(*n*-butyl)-amin
F/489	N-Nitroso-piperidin
F/490	N:N′-Dinitroso-piperazin
F/491	N:N′-Dinitroso-pentamethylen-tetramin
F/505	2:6-Dinitroso-resorzin
F/506	2:6-Dinitroso-5-methyl-resorzin
F/507	2:6-Dinitroso-4-*n*-hexyl-resorzin
F/512	1-Nitroso-2-naphtol-6-sulfonsäure-Na
F/513	2-Nitroso-1-naphtol-4-sulfonsäure-Na
F/514	1-Nitroso-2-naphtol-3:6-disulfonsäure-Na
F/515	Natrium-1-nitroso-2-naphtyl-diazotat
F/516	5-Nitroso-barbitursäure (= Violursäure)
F/519	*iso*Nitroso-aceton
F/520	*n*-Butyraldoxym
F/521	Benzaldoxym
F/523	4-Hydroxy-benzaldoxym
F/524	Furfuraldoxym
F/525	Heptanon-2-oxym
F/526	Cyklopentanon-oxym
F/527	Cyklohexanon-oxym
F/528	*l*-Menthon-oxym
F/529	*d*-Campher-oxym
F/530	Acetophenon-oxym
F/531	Dimethyl-glioxym
F/532	1:4-Benzochinon-dioxym
F/533	1:4-Benzochino-1-oxym-4-aminoguanidon hydrochlorid
F/534	2-Hydroxy-3-nitroso-1:4-benzochinon-1-oxym-4-aminoguanidon hydrochlorid
F/535	2-Hydroxy-3-nitroso-5-*n*-hexyl-1:4-benzochinon-1-oxym-4-aminoguanidon
F/538	1:2-Naphtochinon-2-oxym-1-aminoguanidon
F/541	2-Methyl-1:10-phenanthrolin
F/542	2:4-Dimethyl-1:10-phenanthrolin
F/543	2:9-Dimethyl-1:10-phenanthrolin
F/544	N-Aethyl-1:10-phenanthrolinium aethylsulfat
F/545	1:5-Phenanthrolin
F/546	2:6-Dimethyl-1:5-phenanthrolin
F/547	1:8-Phenanthrolin
F/548	2:7-Dimethyl-1:8-phenanthrolin

weniger wirksam erwiesen. Trotz des Umstandes, dass ihre Mehrheit im allgemeinen mehr oder weniger fungistatisch wirkt, kann man doch feststellen, dass unter ihnen ausser dem 8-Hydroxy-chinolin selbst nur deren 5:7-Dihalogen- 5:7-Dinitro- und 7-Brom-5-aryl-azo-Derivate eine hochgradige fungistatische Wirkung besitzen, jedoch mit Ausnahme der Nr. F/480.

Die 5-Aryl-azo-8-hydroxy-chinoline von Nr. F/462–467, welche in ihrem Molekül zur Säuredissoziation geneigte —COOH bzw. —SO_2NH— Gruppe enthalten, bleiben wirkungslos, in Gegensatz zu den solche Gruppe nicht enthaltenden wirksamen Azo-Verbindungen von Nr. F/458-461. Ihre 7-Brom-Derivate (Nr. F/474–479) verfügen zugleich über das gleiche Mass von fungistatischer Wirkung wie die 5-Aryl-azo-7-brom-8-hydroxy-chinoline, die keine —COOH oder —SO_2NH— Gruppe enthalten (Nr. F/470–473).

TABELLE 3. DIE FUNGISTATISCHE WIRKUNG VON 8-HYDROXY-CHINOLIN-DERIVATEN, NITROSO-VERBINDUNGEN UND OXYMEN AUF FESTEM MAISCHE-AGAR NÄHRBODEN

Nr.	Verbindungen	*Penicillium simplicissimum*	*Aspergillus niger*	*Trichothecium roseum*	*Candida albicans*	*Saccharomyces cerevisiae*
F/445	8-Hydroxy-chinolin	42	25	55	40	30
F/449	5:7-Dichlor-8-hydroxy-chinolin	20	18	23	20	25
F/450	5:7-Dibrom-8-hydroxy-chinolin	14	10	15	12	12
F/451	5:7-Dijod-8-hydroxy-chinolin	10	10	12	10	10
F/452	5-Chlor-7-jod-8-hydroxy-chinolin	15	15	20	17	18
F/454	5:7-Dinitro-8-hydroxy-chinolin	30	45	33	30	30
F/492	4-Nitroso-dimethylanilin	45	35	40	40	35
F/493	4-Nitroso-diaethylanilin	45	35	32	38	36
F/494	4-Nitroso-diphenylamin	10	10	10	10	10
F/495	1-Nitroso-2-naphtylamin	10	12	20	10	12
F/499	Kupfer-2-nitroso-4-methyl-phenolat	10	10	10	10	10
F/501	Kupfer-2-nitroso-6-methyl-phenolat	10	—	12	12	12
F/502	Kupfer-2-nitroso-4-chlor-phenolat	10	10	10	10	10
F/503	Kupfer-2-nitroso-6-chlor-phenolat	10	—	10	—	10
F/508	1-Nitroso-2-naphtol	20	18	20	20	20
F/509	2-Nitroso-1-naphtol	18	12	20	15	16
F/511	9-Nitroso-10-phenanthrol	12	10	12	12	15
F/517	3:5-Dimethyl-4-nitroso-pyrazol	22	27	22	10	10
F/518	1-Phenyl-3:5-dimethyl-4-nitroso-pyrazol	50	45	40	27	32
F/522	2-Hydroxy-benzaldoxym	13	—	12	10	10
F/536	1:2-Naphtochinon-dioxym	15	—	20	15	15
F/539	2:2'-Dipiridyl	18	15	25	22	22
F/540	1:10-Phenanthrolin hydrochlorid	35	25	40	30	26

Die in der Tabelle stehenden Ziffern bezeichnen die Durchmesser der durch die einzelnen Verbindungen herbeiführten wachstumsgehemmten Gebiete in mm ausgedrückt.

— = Die Verbindung übt auf den angewandte Pilzstamm keine fungistatische Wirkung aus.

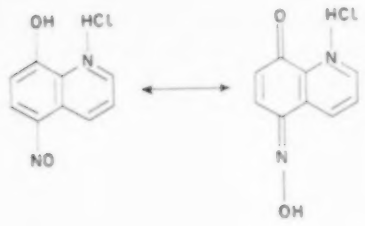
Jene von den untersuchten Verbindungen der Tabelle 1 und 2, welche in der Tabelle 3 nicht aufgeführt sind, übten auf festem Maische-Agar Nährboden keine fungistatische Wirkung auf die zu den Versuchen verwendeten fünf Pilzstämme aus.

Vol. 7
1961

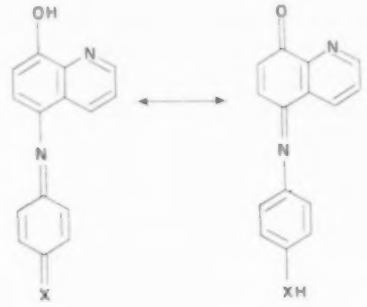
Das Dihydrochlorid des 7-(1′-Piperidino-methyl)-8-hydroxy-chinolins (Nr. F/456) besitzt nur einen niedrigen Wirkungsgrad, sein 5-(4″-Chlor-phenyl-azo-)-Derivat (Nr. F/480) hat aber eine bedeutende Wirksamkeit.

Die aus 8-Hydroxy-chinaldin stammenden Verbindungen von Nr. F/447, 448, 453, 484 und 485 haben wir nur in geringem Masse wirksam gefunden. Die Azo-Verbindungen Nr. F/468, 469 sind wirkungslos. Die Wirkungslosigkeit ist damit zu erklären, dass sie wegen ihrer geringen Lösbarkeit die zur Herbeiführung der Fungistase nötige minimale Konzentration im Nährboden nicht erreichen.

Das 5-Nitroso-8-hydroxy-chinolin hydrochlorid (Nr. F/455) ist nur sehr mässig wirksam, die Verbindungen von Nr. F/481, und 482 sind aber völlig wirkungslos. Die Ursache des geringen Wirkungsgrades der Nr. F/455 und die Wirkungslosigkeit der Nr. F/481 und 482 ist darin zu suchen, dass ihr Aufbau dem Grenzfall der 5-substituierten 8-Hydroxy-chinolin-Struktur und zur Bildung der Chelate-Komplexe unfähigen Chinolin-5:8-chinon-5-oxym, bzw. der Chinolin-5:8-chinon-5-imin Struktur entspricht.



F/455



F/481: X=O
F/482: X=NH

Nicht nur die 8-Hydroxy-chinolin-Derivate, sondern auch die 2-Nitroso-Derivate der Phenole besitzen eine bedeutende Chelate-Komplex-bildende Eigenschaft. Die Kupfer bzw. Zink-Komplexe des 2-Nitroso-phenols und dessen substituierten Derivate (Nr. F/498–504) sind auf flüssigem Nährboden nur auf das apathogene *Trichothecium roseum*, das mausepathogene *Achorion quinckeanum*, das menschenpathogene *Trichophyton gypseum* und das *Epidermophyton Kaufman-Wolff* fungistatisch wirksam, sie wirken jedoch selbst auf diese Pilzstämme nur in einer verhältnissmässig geringen Verdünnung. Die niedere Stufe ihrer fungistatischen Wirkung soll—wenigstens zum Teil—mit ihrer geringen Lösbarkeit im Nährboden in Zusammenhang stehen.

Das 1-Nitroso-2-naphtol (Nr. F/508), das 2-Nitroso-1-naphtol (Nr. F/509) und das 9-Nitroso-10-phenanthrol (Nr. F/511) erwiesen sich als fungistatisch besonders wirksam.

Das zur Chelate-Komplex-Bildung unfähige 4-Nitroso-phenol (Nr. F/497) und das 4-Nitroso-1-naphtol (Nr. F/510) sind zwar fungistatisch wirksam, ihr Wirkungsgrad ist aber verhältnismässig nur von geringem Masse. Ihr Wirkungsmechanismus ist

auch vermutlich ganz verschiedenartig, als derjenige der zur Chelate-Komplex-Bildung fähigen Nitroso-phenole und Nitroso-naphtole.

Das 2:6-Dinitroso-resorzin (Nr. F/505) und dessen Alkyl-Derivate (Nr. F/506, 507), sowie ihre Mono-aminoguanidone (Nr. F/534, 535), ferner die 5-Nitroso-barbitursäure (Nr. F/516) verfügen zwar über eine Chelate-Komplex-bildende Eigenschaft, sind aber fungistatisch wirkungslos. Die Ursachen ihrer Wirkungslosigkeit sind uns unbekannt. Ebenso wirkungslos sind die Sulfonsäure-Salze der Nitroso-naphtole und auch das Natrium-1-nitroso-2-naphtyl-diazotat (Nr. F/512–515). Die Ursache ihrer Wirkungslosigkeit liegt wohl darin, dass wegen des niedrigen Verteilungsquotienten zwischen Lipoid und Wasser diese Verbindungen die aus Lipoproteiden und Chitin bestehende Zellenwand der Pilze nicht durchdiffundieren können.

Die von uns untersuchten N-Nitroso-amine, C-Nitroso-arylamine—mit Ausnahme der Nr. F/495—sowie die 4-Nitroso-pyrazole haben keine Chelate-Komplex-bildende Eigenschaft. Unter diesen verfügen einzelne (Nr. F/486, 494, 496) über eine mässige, andere dagegen (Nr. F/492, 493, 517, 518) über eine beträchtliche fungistatische Wirksamkeit. Ihr Wirkungsmechanismus ist offenbar ganz andersartig, als diejenige der zur Chelate-Komplex-Bildung fähigen Nitroso-phenole. In der Reihe der untersuchten C-Nitroso-arylaminen besitzt eine Verbindung, das 1-Nitroso-2-naphtylamin (Nr. F/495) Chelate-Komplex-bildende Fähigkeit und zugleich auch eine mässige fungistatische Wirkung. Ihr Wirkungsmechanismus ist vermutlich ähnlich als derjenige der Nitroso-naphtole.

In der Reihe der untersuchten Aldoxyme, Ketoxyme, Chinonoxyme (und der Aminoguanidone der letzteren) sind nur das 2-Hydroxy-benaldoxym (Nr. F/522), das 1:2-Naphtochinon-dioxym (Nr. F/536), das 1:2-Naphtochinon-1-oxym-2-aminoguanidon (Nr. F/537), das 1:2-Naphtochinon-2-oxym-1-aminoguanidon (Nr. F/538) und das Dimethyl-glioxym (Nr. F/531) zur Chelate-Komplex-Bildung geneigt; die letzten zwei Verbindungen sind wirkungslos, während die ersten drei Verbindungen fungistatisch sich als mässig wirksam erweisen.

Das zum Vergleich untersuchte 2:2′-Dipiridyl (Nr. F/539) und das 1:10-Phenanthrolin hydrochlorid (Nr. F/540)—welche in starkem Masse Chelate-Komplex-bildend sind—zeigten sich fungistatisch beträchtlich wirksam. Einige von uns untersuchten Derivate des 1:10-Phenanthrolins, sowie seine Isomere—die zur Bildung von Chelate-Komplexen unfähig sind (Nr. F/541–548)—bekundeten dagegen eine gänzliche Wirkungslosigkeit.

Aus dem Durchblick der Ergebnisse der Tabelle 3, kann man ersehen, dass unter den untersuchten Verbindungen auf festem Nährboden sich viel wenigere fungistatisch wirkend gefunden wurden, als auf einem flüssigen Kulturmedium. Diese Erscheinung lässt sich wohl darauf zurückführen, dass die Diffusion der Mehrheit der untersuchten Verbindungen im festen Nährboden—wegen ihrer bedeutenden Molekülausmasse und infolge ihrer geringen Lösbarkeit—nur einen geringen Grad erreichte, oder aber vermochten sie überhaupt nicht in den Nähr-Agar zu diffundieren.

UNTERSUCHUNGEN ÜBER DEN WIRKUNGSMECHANISMUS

Indem das 8-Hydroxy-chinolin und seine Derivate—an Hand der bisherigen Erfahrungen—in Bezug auf ihre biologischen Auswirkungen qualitative im allgemeinen

keine beachtungswerte Organismus-Spezifität aufweisen, so kann man mit Recht voraussetzen, dass sie ihre fungistatische Wirkung wohl auf Grund eines ähnlichen Mechanismus ausüben müssen, wie ihre bakteriostatische Wirkung.

Zahlreiche Autoren haben nachgewiesen, dass die bakteriostatische Wirkung des 8-Hydroxy-chinolins und einiger Derivate dessen durch einige Schwermetall-Ione abgewehrt werden kann.[21-32]

Daraus kann man den Schluss ziehen, dass in dem biochemischen Mechanismus ihrer bakteriostatischen Wirkung auf irgendwelche Weise —mittelbar oder unmittelbar —ihre Chelate-Komplex-bildende Fähigkeit entscheidend mitwirken soll.

Nach den Erfahrungen von Zentmyer[47] sowie Uri und Szabó[5] kann nicht nur die bakteriostatische, sondern auch die fungistatische Wirkung des 8-Hydroxy-chinolins durch einzelne Schwermetall-Ione abgewehrt werden. Daraus kann man die Folgerung ziehen, dass der biochemische Mechanismus der fungistatischen Wirkung des 8-Hydroxy-chinolins mehr oder weniger ähnlich sein kann, als der Wirkungsmechanismus seiner bakteriostatischen Wirkung.

Jeney und Zsolnai haben den Beweis erbracht, dass einzelne Schwermetall-Ione nicht nur die bakteriostatische Wirkung des 8-Hydroxy-chinolins zu beeinflussen vermögen, sondern auch dieselbe der 10-Hydroxy-phenanthroline, der Nitroso-naphtole und des 2:2′-Dipiridyls.[25-28]

Wenn man dies berücksichtigt, scheint es naheliegend zu sein, dass die Fähigkeit der Chelate-Komplex-Bildung nicht nur im Wirkungsmechanismus des 8-Hydroxy-chinolins und einiger Derivate desselben eine entscheidende Rolle spielen sollte, sondern auch in dem Wirkungsmechanismus von Verbindungen anderen Typus wesentlich mitwirken mag.

Nach Rubbo, Albert and Gibson übt das 8-Hydroxy-chinolin (und auch seine aktiven Derivate) ihre bakteriostatische Wirkung dadurch aus, dass es mit den Schwermetall-Ionen im Nährboden Chelate-Komplexe bilden, welche die Weg-Oxidierung der SH-Radikale einzelner lebenswichtiger Sulfhydryl-Enzyme der Bakterien katalisieren.[22, 23]

Wenn das 8-Hydroxy-chinolin und dessen einzelne Derivate ihren fungistatischen Einfluss wirklich durch indirekte, d.h. durch gewissen Metall-Chelate-Komplexe erfolgte Destruktion der Sulfhydryl-Enzyme der Pilzzellen bewirken, so muss in diesem Falle diese fungistatische Wirkung nicht nur durch einzelne Schwermetall-Ione, sondern auch durch reduzierende Stoffe abgewehrt werden können, welche die Fort-Oxidierung der SH-Radikale verhindern.

Im Laufe unserer Wirkungsmechanismus-Untersuchungen haben wir folgende Probleme studiert:

(a) Welche Schwermetall-Ione vermögen die fungistatische Wirkung einiger von uns am wirksamsten gefundener Chelate-Komplex-bildenden Verbindungen sowohl auf flüssigem als auch auf festem Maische-Nährboden abzuwehren?

(b) Inwiefern deren fungistatische Wirksamkeit, sowie die Beeinflussbarket derselben infolge einzelner Schwermetall-Ione durch die Gegenwart von Serum im Nährboden beeinflusst werden kann?

(c) Ob jene Schwermetall-Ione, welche ihre fungistatische Wirkung auf flüssigem Nährboden abwehren können, die durch sie bewirkte Fungistase nicht nur abzuwehren, sondern auch aufzuheben vermögen.

(d) Ob reduzierende Stoffe, wie Cystein hydrochlorid, Natrium-thioglycolat und Ascorbinsäure auf festem Nährboden die fungistatische Wirkung der 8-Hydroxy-chinolin-Derivate abzuwehren imstande sind?

Indem es anzunehmen war, dass einige andere von uns untersuchte Verbindungen mit Chelate-Komplex-bildender Eigenschaft und auch mit einer bedeutenden fungistatischen Wirkung ihren fungistatischen Einfluss auf Grund eines mehr oder ähnlichen Mechanismus ausüben, wie das 8-Hydroxy-chinolin uns dessen Derivate—obwohl sie in Bezug auf ihre chemische Struktur von den vorigen und auch voneinander bedeutend abweichen, jedoch betreffs einer Eigenschaft, nähmlich ihre Fähigkeit um Schwermetall-Ione zu chelatieren, einander ähnlich sind—deshalb haben wir die in den vorangehenden a.-d. Punkten erwähnten Untersuchungen auch auf diese ausgedehnt.

Die Nitroso-Verbindungen von Nr. F/492, 493, 517 und 518 besitzen zwar keine Chelate-Komplex-bildende Fähigkeit, sie erwiesen sich jedoch im Laufe unserer Untersuchungen fungistatisch als beträchtlich wirksam. Ihr Wirkungsmechanismus ist offenbar ganz andersartig, als derselber der Chelate-Komplex-bildenden Verbindungen, deshalb haben wir die in dem a.-c. Punkten angeführten Untersuchungen mit ihnen nicht durchgeführt. Wegen der Reaktivität ihrer Moleküle sind sie immerhin vielseitiger chemischer Reaktionen fähig, so kann es vorausgesetzt werden, dass sie mit einzelnen Sulfhydryl-Enzymen entweder auf Grund eines Kondensations-oder gar eines einfachen oxydativen Mechanismus reagieren und jene dadurch inaktivieren können und dass sie ihre fungistatische Wirkung entweder vermittels eines solchen oder diesem ähnlichen Mechanismus ausüben imstande sind.

Indem diese unsere Annahme sich als stichhaltig erweist, so müssen Verbindungen mit Sulfhydryl-Radikal (oder eventuell auch andere Reduktionsmittel) ihre fungistatischen Wirkung abwehren können. Deshalb haben wir die im Punkt d. erwähnten Untersuchungen auch bei den Verbindungen von Nr. F/492, 493, 517 und 518 durchgeführt.

Für unsere Versuche über den Wirkungsmechanismus haben wir als Test-Organismus *Penicillium simplicissimum* und *Saccharomyces cerevisiae* angewandt. Das pH des serumfreien als auch des serumhaltigen flüssigen und festen Maische-Nährbodens, sowie des Cystein-hydrochlorid, Natriumthioglocolat, Ascorbinsäure, bzw. Schwermetallsalze enthaltenden festen Maische-Nährbodens betrug gleicherweise 6,0–6,2.

A. UNTERSUCHUNGSMETHODE

Aus den Prüfgläsern, die 5 ml Maische- bzw. Serum-Maische-Nährboden enthielten· haben wir—bei strenger Beobachtung der Regeln der Sterilität—mit einer Pipette Mengen von 1, 0,5, 0,2, 0,1 ml ausgenommen, dann haben wir an deren statt die 0,5 0,25, 0,1, 0,05 ml Mengen der Wässerigen Lösungen von Na-Salzen bzw. Hydrochloriden der untersuchten Verbindungen von M/100 bzw. M/1000 Konzentration und ähnliche Mengen aus den Wässerigen Lösungen von M/50 bzw. M/500 Konzentration der zu den Untersuchungen verwendeten Schwermetall-Salze eingemessen. In die Prüfgläser, in welche keine Metall-Salz-Lösungen gegeben wurden, welche also bloss die untersuchte Chela te-Komplex-bildende Verbindung enthielten, haben wir

Vol. 7
1961

statt der Metall-Salzlösung die entsprechende Menge sterilen destillierten Wassers gegeben.

So waren die untersuchten Verbindungen in den einzelnen Prüfgläsern in M/1000, M/2000, M/5000, M/10 000, M/20 000, M/50 000, M/100 000 Konzentration, die zu der Untersuchung angewandten Schwermetall-Salze dagegen in M/500, M/1000, M/2500, M/5000, M/10 000, M/25 000 und M/50 000 Konzentration anwesend. Es fielen also 2 mol Schwermetall-Ione gleicherweise in einer jeden Konzentration auf 1 mol Chelate-Komplex-bildende Verbindung.

Danach haben wir die Prüfgläser mit der Sporensuspension des *Penicillium simplicissimum* bzw. des *Saccharomyces cerevisiae* infiziert und darauf durch 5 Tage in einem Thermostat von 30 °C inkubieren lassen. Nach Verlauf der Inkubationszeit haben wir die Ergebnisse an Hand des Wachstums im Vergleich zu den Kontrollen ausgewertet.

Die zu den Versuchen angewandten Schwermetall-Salze haben an sich allein selbst in M/500 Konzentration die Vermehrung weder des *Penicillium simplicissimum* noch des *Saccharomyces cerevisiae* beeinflussen können.

Andere Einzelheiten der Untersuchungsmethode (z.B. die Zahl der Parallele, die Zubereitung der zur Inokulation notwendigen Sporensuspension, die Temperatur usw.) waren gänzlich identisch mit denen, die im ersten Teil unserer Mitteilungsserie beschrieben wurden.[1]

Die Resultate dieser Untersuchungen haben bloss gezeigt, ob irgendein Schwermetall-Ion, das dem Nährboden mit dem wirksamen Agens nahezu gleichzeitig zugegeben wurde, imstande sein kann, die fungistatische Wirkung dieses Agens abzuwehren, die er auf das—zum System später zugegebene-*Penicillium simplicissimum* bzw. *Saccharomyces cerevisiae* ausübt. Deshalb nennen wir diese Versuche "*Abwehrversuche*".

Die Abwehr der fungistatischen Wirkung der Chelate-Komplex-bildenden Verbindungen durch irgendein Schwermetall-Ion mag auch die Folge eines einfachen extrazellularen Vorganges sein. Z.b. die Lösbarkeit des Komplexes irgendeiner Chelate-Komplex-bildenden Verbindung, die mit einem Schwermetall-Ion extrazellular gebildet wurde, vermag sehr niedrigen Grades zu sein und dadurch kann die Konzentration des Agens bzw. des Agens-Schwermetall-Komplexes im Nährboden unter die Grenzkonzentration von fungistatischer Wirkung sinken. Wegen dieser Umstände können zwar Versuche dieser Art in Bezug auf den Wirkungsmechanismus der Chelate-Komplex-bildenden Vergindungen einen informativen Wert besitzen, sie bringen aber doch kein sicheres Ergebnis für uns.

Mit Rücksicht darauf haben wir ausser den "*Abwehrversuchen*" auch "*Aufhebungsversuche*" ausgeführt, im Verlaufe derjenigen wir nur insofern von der vorher berichteten Methode abgewichen waren, dass wir die Nährböden nach Einmessen der untersuchten Verbindungen infizierten und zu den Prüfgläsern, worin wir nach 5 Tage Inkubationsdauer eine gänzliche Fungistase beobachteten, haben wir *erst danach* die Schwermetall-Salzlösungen von entsprechender Konzentration zugegeben. Die Ergebnisse haben wir nach einer weiteren Inkubation von 5 Tagen ausgewertet, an Hand ihres Vergleiches mit den Kontrollen, die zwar gleicherweise zubereitet, doch anstatt der Metall-Salzlösungen eine entsprechende Menge steriles destilliertes Wasser enthielten und gleichfalls eine Gesamt-Inkubationszeit von 10 Tagen benötigten.

Zur Entscheidung der Frage, ob die fungistatische Wirkung der untersuchten Chelate-Komplex-bildenden Verbindungen im Laufe dieser Versuche durch irgendein Schwermetall-Ion nur deshalb nicht aufgehoben werden konnte, da sie eventuell während der Einwirkungsdauer auf das *Penicillium simplicissimum* oder auf das *Saccharomyces cerevisiae* eine *fungizide* Wirkung ausübte, haben wir je eine Menge von 0,1 ml—vor der Zugabe der Metall-Salze zu den Prüfgläsern, die die untersuchten Verbindungen enthielten, jedoch von einer Pilzentwicklung frei waren—aus den einzelnen Prüfgläsern mit Pipette (aus einem jeden Glas mit anderer Pipette!) herausgenommen, diese Menge zu 5 ml Maische-Nährboden gegeben und *erst danach* die entsprechenden Mengen der Schwermetall-Salzlösungen ihnen zugegeben.

Die auf diese Weise "infizierten" Prüfgläser haben wir für 5 Tage in einen Thermostat von 30 °C Temperatur gestellt und die in ihnen erfolgte Entwicklung des *Penicillium simplicissimum* und des *Saccharomyces cerevisiae* nach dieser Inkubationsdauer abgelesen.

Indem in den Prüfgläsern, dessen Gehalt aus jenen Prüfgläsern stammte, welche die untersuchten Verbindungen in M/1000 Konzentration nach 5 tägiger Inkubationszeit enthielten, in 5 Tagen die Entwicklung des *Penicillium simplicissimum* oder des *Saccharomyces cerevisiae* beobachtet werden konnte, haben wir es als bestätigt erachtet, dass die untersuchte Verbindung in M/1000 Konzentration während der 5 Tage Dauer ihrer Einwirkung auf die erwähnten Pilzstämme *fungizid* wirkungslos blieb; also wenn irgendein Schwermetall-Ion deren fungistatische Wirkung nicht aufhebt, so ist es nicht daraúf zurückzuführen, dass die betreffende Verbindung die Pilzsporen während 5 Tage tötete und deren Wirkung somit *schon vornherein* nicht aufgehoben werden kann, sondern lässt sich eben dem Umstand zuschreiben, dass sie in den Pilzsporen diejenigen durch die Verbindung bewirkten intrazellularen biochemischen Veränderungen nicht mehr reversibel zu machen vermag, infolge deren die Vermehrung des *Penicillium simplicissimum* und des *Saccharomyces cerevisisiae* gehemmt wurde—welche biochemische Veränderung jedoch die Zelle nicht tötete, sondern nur deren Vermehrung vorübergehend hemmte.

Also während wir den im Laufe der "*Abwehrversuche*" bewirkten hemmenden Einfluss der untersuchten Chelate-Komplex-bildenden Verbindungen auf die Vermehrung des *Penicillium simplicissimum* und des *Saccharomyces cerevisiae* mittels der verschiedenen Schwermetall-Ione *eventuell nur extrazellular* beeinflussen konnten, so liefern die Ergebnisse unserer "*Aufhebungsversuche*" auch darauf Antwort, ob deren fungistatische Wirkung durch Schwermetall-Ione auch *intrazellular* beeinflusst werden kann, d.h. durch diese Agenzien *auch in der Zelle herbeigeführten* biochemischen Veränderungen durch die erwähnten Schwermetall-Ione reversibel gemacht werden könnten.

"*Abwehrsversuche*" haben wir nicht nur auf flüssigem, sondern auch auf festem Nährboden ausgeführt.

Zu diesen unseren Untersuchungen haben wir in 0,1% Schwermetall-Salze, bzw. in 0,3% Cystein-hydrochlorid, Natrium-thioglycolat, bzw. Askorbinsäure enthaltende Maische-Agar Nöhrböden verwendet.

B. ERGEBNISSE

Die Resultate unserer Untersuchungen über den Wirkungsmechanismus haben wir in den Tabellen 4, 5, 6, 7, 8 zusammengefasst.

TABELLE 4. DIE ABWEHRUNG DER FUNGISTATISCHEN WIRKUNG CHELATE-KOMPLEX-BILDENDER VERBINDUNGEN DURCH SCHWERMETALL-IONE AUF FLÜSSIGEM NÄHRBODEN

Nr.	Verbindungen	Die zum Nährboden hinzugefügten Schwermetallione	Die fungistatisch wirkende molare Grenzkonzentration der Verbindung			
			auf Maische-Nährboden	auf 10% Rinderserum enthaltendem Maische-Nährboden	auf Maische-Nährboden	auf 10% Rinderserum enthaltendem Maische-Nährboden
			Penicillium simplicissimum		*Saccharomyces cerevisiae*	
F/445	8-Hydroxy-chinolin	—	M/ 20 000	M/ 20 000	M/ 20 000	M/ 20 000
		Cu^{2+}	M/100 000	M/100 000	M/100 000	M/100 000
		Fe^{2+}	—	—	—	—
		Zn^{2+}	M/ 20 000	M/ 20 000	M/ 20 000	M/ 20 000
		Mn^{2+}	M/ 20 000	M/ 20 000	M/ 20 000	M/ 20 000
		Co^{2+}	—	—	—	—
		MoO_4^{2-}	M/ 10 000	M/ 10 000	M/ 10 000	M/ 10 000
F/449	5:7-Dichlor-8-hydroxy-chinolin	—	M/ 50 000	M/ 50 000	M/ 50 000	M/ 50 000
		Cu^{2+}	—	—	—	—
		Fe^{2+}	—	—	—	—
		Zn^{2+}	M/ 50 000	M/ 50 000	M/ 50 000	M/ 50 000
		Mn^{2+}	M/ 50 000	M/ 50 000	M/ 50 000	M/ 50 000
		Co^{2+}	M/ 10 000	M/ 5000	M/ 10 000	M/ 5000
		MoO_4^{2-}	M/ 50 000	M/ 20 000	M/ 50 000	M/ 50 000
F/450	5:7-Dibrom-8-hydroxy-chinolin	—	M/ 50 000	M/ 50 000	M/ 50 000	M/ 50 000
		Cu^{2+}	—	—	—	—
		Fe^{2+}	—	—	—	—
		Zn^{2+}	M/ 50 000	M/ 50 000	M/ 50 000	M/ 50 000
		Mn^{2+}	M/ 50 000	M/ 50 000	M/ 50 000	M/ 50 000
		Co^{2+}	M/ 5000	M/ 1000	M/ 5000	M/ 1000
		MoO_4^{2-}	M/ 20 000	M/ 20 000	M/ 20 000	M/ 20 000
F/451	5:7-Dijod-8-hydroxy-chinolin	—	M/ 20 000	M/ 20 000	M/ 20 000	M/ 20 000
		Cu^{2+}	M/ 1000	M/ 1000	M/ 1000	M/ 1000
		Fe^{2+}	—	—	—	—
		Zn^{2+}	M/ 20 000	M/ 20 000	M/ 20 000	M/ 20 000
		Mn^{2+}	M/ 20 000	M/ 20 000	M/ 20 000	M/ 20 000
		Co^{2+}	M/ 1000	M/ 1000	M/ 2000	M/ 2000
		MoO_4^{2-}	M/ 10 000	M/ 10 000	M/ 2000	M/ 2000
F/452	5-Chlor-7-jod-8-hydroxy-chinolin	—	M/ 50 000	M/ 50 000	M/ 50 000	M/ 50 000
		Cu^{2+}	—	—	—	—
		Fe^{2+}	—	—	—	—
		Zn^{2+}	M/ 50 000	M/ 50 000	M/ 50 000	M/ 50 000
		Mn^{2+}	M/ 50 000	M/ 50 000	M/ 20 000	M/ 20 000
		Co^{2+}	M/ 2000	—	M/ 2000	—
		MoO_4^{2-}	M/ 10 000	M/ 10 000	M/ 10 000	M/ 10 000
F/454	5:7-Dinitro-8-hydroxy-chinolin	—	M/ 50 000	M/ 20 000	M/ 20 000	M/ 20 000
		Cu^{2+}	—	—	—	—
		Fe^{2+}	—	—	—	—
		Zn^{2+}	M/ 1000	—	M/ 1000	—
		Mn^{2+}	M/ 50 000	M/ 20 000	M/ 20 000	M/ 20 000
		Co^{2+}	—	—	—	—
		MoO_4^{2-}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
F/495	1-Nitroso-2-naphtylamin	—	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Cu^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Fe^{2+}	—	—	—	—
		Zn^{2+}	M/ 2000	M/ 2000	M/ 1000	M/ 1000
		Mn^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Co^{2+}	—	—	—	—
		MoO_4^{2-}	M/ 2000	M/ 2000	M/ 2000	M/ 2000

Vol. 7
1961

TABELLE 4—*fortges.*

Nr.	Verbindungen	Die zum Nährboden hinzugefügten Schwermetallione	Die fungistatisch wirkende molare Grenzkonzentration der Verbindung			
			auf Maische-Nährboden	auf 10% Rinderserum enthaltendem Maische-Nährboden	auf Maische-Nährboden	auf 10% Rinderserum enthaltendem Maische-Nährboden
			Penicillium simplicissimum		*Saccharomyces cerevisiae*	
F/508	1-Nitroso-2-naphtol	—	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Cu^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Fe^{2+}	—	—	—	—
		Zn^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Mn^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Co^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		MoO_4^{2-}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
F/509	2-Nitroso-1-naphtol	—	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Cu^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Fe^{2+}	—	—	—	—
		Zn^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Mn^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Co^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		MoO_4^{2-}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
F/511	9-Nitroso-10-phenanthrol	—	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Cu^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Fe^{2+}	—	—	—	—
		Zn^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Mn^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Co^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		MoO_4^{2-}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
F/536	1:2-Naphtochinon-dioxym	—	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Cu^{2+}	M/ 1000	M/ 1000	M/ 1000	M/ 1000
		Fe^{2+}	—	—	—	—
		Zn^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Mn^{2+}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
		Co^{2+}	M/ 1000	M/ 1000	M/ 1000	M/ 1000
		MoO_4^{2-}	M/ 2000	M/ 2000	M/ 2000	M/ 2000
F/539	2:2'-Dipiridyl	—	M/ 5000	M/ 5000	M/ 5000	M/ 5000
		Cu^{2+}	—	—	—	—
		Fe^{2+}	—	—	—	—
		Zn^{2+}	—	—	—	—
		Mn^{2+}	M/ 5000	M/ 5000	M/ 5000	M/ 5000
		Co^{2+}	—	—	—	—
		MoO_4^{2-}	M/ 5000	M/ 5000	M/ 5000	M/ 5000
F/540	1:10-Phenanthrolin hydrochlorid	—	M/ 20 000	M/ 20 000	M/ 20 000	M/ 20 000
		Cu^{2+}	—	—	—	—
		Fe^{2+}	—	—	—	—
		Zn^{2+}	—	—	—	—
		Mn^{2+}	M/ 20 000	M/ 20 000	M/ 20 000	M/ 20 000
		Co^{2+}	—	—	—	—
		MoO_4^{2-}	M/ 20 000	M/ 20 000	M/ 20 000	M/ 20 000

— = Die untersuchte Verbindung übt in der Anwesenheit des betreffenden Schwermetall-Ions—unter den angewandten Versuchsbedingungen—noch in der Konzentration von M/1000 keine fungistatische Wirkung aus.

Vol. 7
1961

TABELLE 5. DIE ABWEHRUNG DER FUNGISTATISCHEN WIRKUNG CHELATE-KOMPLEX-BILDENDER VERBINDUNGEN DURCH SCHWERMETALL-SALZE AUF FESTEN NÄHRBODEN

Nr.	Verbindungen	Die Durchmesser der wachstumsgehemmten Gebiete in mm ausgedrückt auf Maische-Agar Nährboden						
		Penicillium simplicissimum						
		(ohne Zugaben)	$CuSO_4.5H_2O$	$FeSO_4.7H_2O$	$ZnSO_4.7H_2O$	$MnCl_2.4H_2O$	$Co(NO_3)_2.6H_2O$	$(NH_4)_2MoO_4$
F/445	8-Hydroxy-chinolin	42	18	10	—	42	—	—
F/449	5:7-Dichlor-8-hydroxy-chinolin	20	—	—	20	17	—	—
F/450	5:7-Dibrom-8-hydroxy-chinolin	14	—	—	10	13	—	—
F/452	5-Chlor-7-jod-8-hydroxy-chinolin	15	—	—	15	15	—	—
F/454	5:7-Dinitro-8-hydroxy-chinolin	30	—	—	—	32	—	—
F/508	1-Nitroso-2-naphtol	20	—	—	20	20	—	19
F/509	2-Nitroso-1-naphtol	18	—	—	17	15	—	18
F/511	9-Nitroso-10-phenanthrol	12	—	—	11	13	—	13
F/536	1:2-Naphtochinon-dioxym	15	—	—	14	14	—	12
F/539	2:2′-Dipiridyl	18	—	—	—	17	—	—
F/540	1:10-Phenanthrolin hydrochlorid	35	—	—	—	20	—	—

Nr.	Verbindungen	Die Durchmesser der wachstumsgehemmten Gebiete in mm ausgedrückt auf Maische-Agar Nährboden						
		Saccharomyces cerevisiae						
		——	$CuSO_4.5H_2O$	$FeSO_4.7H_2O$	$ZnSO_4.7H_2O$	$MnCl_2.4H_2O$	$Co(NO_3)_2.6H_2O$	$(NH_4)_2MoO_4$
F/445	8-Hydroxy-chinolin	30	20	10	—	31	—	—
F/449	5:7-Dichlor-8-hydroxy-chinolin	25	—	—	20	20	—	—
F/450	5:7-Dibrom-8-hydroxy-chinolin	12	—	—	12	10	—	—
F/452	5-Chlor-7-jod-8-hydroxy-chinolin	18	—	—	20	20	—	—
F/454	5:7-Dinitro-8-hydroxy-chinolin	30	—	—	—	28	—	—
F/508	1-Nitroso-2-naphtol	20	—	—	19	20	—	17
F/509	2-Nitroso-1-naphtol	16	—	—	15	16	—	15
F/511	9-Nitroso-10-phenanthrol	15	—	—	13	16	—	16
F/536	1:2-Naphtochinon-dioxym	15	—	—	15	14	—	11
F/539	2:2′-Dipiridyl	22	—	—	—	20	—	—
F/540	1:10-Phenanthrolin hydrochlorid	26	—	—	—	21	—	—

TABELLE—5 *fortges.*

Nr.	Verbindungen	Die Durchmesser der wachstumsgehemmten Gebiete in mm ausgedrückt auf Serum-Maische-Agar Nährboden						
		Penicillium simplicissimum						
		(ohne Zugaben)	$CuSO_4.5H_2O$	$FeSO_4.7H_2O$	$ZnSO_4.7H_2O$	$MnCl_2.4H_2O$	$Co(NO_3)_2.6H_2O$	$(NH_4)_2MoO_4$
F/445	8-Hydroxy-chinolin	42	10	10	—	40	—	—
F/449	5:7-Dichlor-8-hydroxy-chinolin	20	—	—	17	17	—	—
F/450	5:7-Dibrom-8-hydroxy-chinolin	14	—	—	10	10	—	—
F/452	5-Chlor-7-jod-8-hydroxy-chinolin	15	—	—	13	15	—	—
F/454	5:7-Dinitro-8-hydroxy-chinolin	30	—	—	—	25	—	—
F/508	1-Nitroso-2-naphtol	20	—	—	18	17	—	20
F/509	2-Nitroso-1-naphtol	18	—	—	18	18	—	18
F/511	9-Nitroso-10-phenanthrol	12	—	—	12	13	—	13
F/536	1:2-Naphtochinon-dioxym	15	—	—	14	15	—	14
F/539	2:2′-Dipiridyl	18	—	—	—	15	—	—
F/540	1:10-Phenanthrolin hydrochlorid	35	—	—	—	22	—	—

Nr.	Verbindungen	Die Durchmesser der wachstumsgehemmten Gebiete in mm ausgedrückt auf Serum-Maische-Agar Nährboden						
		Saccharomyces cerevisiae						
		———	$CuSO_4.5H_2O$	$FeSO_4.7H_2O$	$ZnSO_4.7H_2O$	$MnCl_2.4H_2O$	$Co(NO_3)_2.6H_2O$	$(NH_4)_2.MoO_4$
F/445	8-Hydroxy-chinolin	30	18	12	—	30	—	—
F/449	5:7-Dichlor-8-hydroxy-chinolin	25	—	—	17	19	—	—
F/450	5:7-Dibrom-8-hydroxy-chinolin	12	—	—	10	10	—	—
F/452	5-Chlor-7-jod-8-hydroxy-chinolin	18	—	—	20	19	—	—
F/454	5:7-Dinitro-8-hydroxy-chinolin	30	—	—	—	26	—	—
F/508	1-Nitroso-2-naphtol	20	—	—	18	18	—	20
F/509	2-Nitroso-1-naphtol	16	—	—	16	15	—	15
F/511	9-Nitroso-10-phenanthrol	15	—	—	13	13	—	13
F/536	1:2-Naphtochinon-dioxym	15	—	—	12	13	—	12
F/539	2:2′-Dipiridyl	22	—	—	—	20	—	—
F/540	1:10-Phenanthrolin hydrchlorid	26	—	—	—	20	—	—

— = Die Verbindung übt auf den Pilzstamm—unter den angewandten Versuchsbedingungen—keine fungistatische Wirkung aus

TABELLE 6. DIE AUFHEBBARKEIT DER FUNGISTATISCHEN WIRKUNG CHELATE-KOMPLEX-BILDENDER VERBINDUNGEN DURCH SCHWERMETALL-IONE AUF FLÜSSIGEN NÄHRBÖDEN

Nr.	Verbindungen	Die zum Nährboden himzugefügten Schwermetall-Ione	Die fungistatisch wirkende molare Grenzkonzentration der Verbindung							
			auf Maische-Nährboden				auf 10% Rinderserum ehtlatendem Maische-Nährboden			
			nach 5 tägiger Inkubationszeit		nach 10 tägiger Inkubationszeit		nach 5 tägiger Inkubationszeit		nach 10 tägiger Inkubationszeit	
			Penicillium simplicissimum	*Saccharomyces cerevisiae*	*Penicillium simplicissimum*	*Saccharomyces cerevisiae*	*Penicillium simplicissimum*	*Saccharomyces cerevisiae*	*Penicillium simplicissimum*	*Saccharomyces cerevisiae*
F/445	8-Hydroxy-chinolin	—	M/20 000	M/20 000	M/20 000	M/10 000	M/20 000	M/20 000	M/20 000	M/20 000
		Fe^{2+}			M/ 5000	M/ 2000			M/ 5000	M/ 5000
		Co^{2+}			M/20 000	M/ 5000			M/20 000	M/20 000
		MoO_4^{2-}			M/ 2000	M/ 2000			M/ 2000	M/ 2000
F/449	5:7-Dichlor-8-hydroxy-chinolin	—	M/50 000	M/50 000	M/50 000	M/50 000	M/50 000	M/50 000	M/50 000	M/50 000
		Cu^{2+}			M/ 2000	M/ 1000			M/ 2000	M/ 1000
		Fe^{2+}			M/ 2000	M/ 1000			M/ 2000	M/ 1000
		Co^{2+}			M/50 000	M/50 000			M/50 000	M/50 000
		MoO_4^{2-}			M/50 000	M/50 000			M/50 000	M/50 000
F/450	5:7-Dibrom-8-hydroxy-chinolin	—	M/50 000	M/50 000	M/50 000	M/50 000	M/50 000	M/50 000	M/50 000	M/50 000
		Cu^{2+}			—	—			—	—
		Fe^{2+}			M/ 2000	M/ 1000			M/ 1000	M/ 1000
		Co^{2+}			M/50 000	M/20 000			M/50 000	M/50 000
		MoO_4^{2-}			M/50 000	M/50 000			M/50 000	M/50 000
F/451	5:7-Dijod-8-hydroxy-chinolin	—	M/20 000	M/20 000	M/20 000	M/20 000	M/20 000	M/20 000	M/20 000	M/20 000
		Cu^{2+}			—	—			—	—
		Fe^{2+}			M/ 1000	M/ 1000			M/ 1000	M/ 1000
		Co^{2+}			M/20 000	M/20 000			M/20 000	M/20 000
		MoO_4^{2-}			M/20 000	M/20 000			M/20 000	M/20 000
F/452	5-Chlor-7-jod-8-hydroxy-chinolin	—	M/50 000	M/50 000	M/50 000	M/50 000	M/50 000	M/50 000	M/50 000	M/50 000
		Cu^{2+}			M/ 2000	M/ 2000			M/ 2000	M/ 2000
		Fe^{2+}			M/ 2000	M/ 2000			M/ 2000	M/ 2000
		Co^{2+}			M/50 000	M/50 000			M/50 000	M/50 000
		MoO_4^{2-}			M/50 000	M/50 000			M/50 000	M/50 000
F/454	5:7-Dinitro-8-hydroxy-chinolin	—	M/50 000	M/20 000	M/50 000	M/20 000	M/50 000	M/20 000	M/50 000	M/20 000
		Cu^{2+}			M/10 000	M/20 000			M/10 000	M/10 000

TABELLE 6.—*fortges.*

Nr.	Verbindungen	Die zum Nährboden himzugefügten Schwermetall-Ione	Die fungistatisch wirkende molare Grenzkonzentration der Verbindung							
			auf Maische-Nährboden				auf 10% Rinderserum ehtlatendem Maische-Nährboden			
			nach 5 tägiger Inkubationszeit		nach 10 tägiger Inkubationszeit		nach 5 tägiger Inkubationszeit		nach 10 tägiger Inkubationszeit	
			Penicillium simplicissimum	*Saccharomyces cerevisiae*	*Penicillium simplicissimum*	*Saccharomyces cerevisiae*	*Penicillium simplicissimum*	*Saccharomyces cerevisiae*	*Penicillium simplicissimum*	*Saccharomyces cerevisiae*
F/454 *fortges.*		Fe^{2+} Zn^{2+} Co^{2+} MoO_4^{2-}			M/10 000 M/50 000 M/20 000 M/50 000	M/20 000 M/20 000 M/20 000 M/20 000			M/10 000 M/50 000 M/20 000 M/50 000	M/10 000 M/20 000 M/20 000 M/20 000
F/495	1-Nitroso-2-naphtylamin	— Fe^{2+} Co^{2+}	M/ 2000	M/ 2000	M/ 2000 M/ 2000 M/ 2000	M/ 2000 M/ 2000 M/ 2000	M/ 2000	M/ 2000	M/ 2000 M/ 2000 M/ 2000	M/ 2000 M/ 2000 M/ 2000
F/508	1-Nitroso-2-naphtol	— Fe^{2+}	M/ 2000	M/ 2000	M/ 2000 M/ 2000	M/ 2000 M/ 2000	M/ 2000	M/ 2000	M/ 2000 M/ 2000	M/ 2000 M/ 2000
F/509	2-Nitroso-1-naphtol	— Fe^{2+}	M/ 2000	M/ 2000	M/ 2000 M/ 2000	M/ 2000 M/ 2000	M/ 2000	M/ 2000	M/ 2000 M/ 2000	M/ 2000 M/ 2000
F/511	9-Nitroso-10-phenanthrol	— Fe^{2+}	M/ 2000	M/ 2000	M/ 2000 M/ 2000	M/ 2000 M/ 2000	M/ 2000	M/ 2000	M/ 2000 M/ 2000	M/ 2000 M/ 2000
F/536	1:2-Naphtochinon-dioxym	— Fe^{2+}	M/ 2000	M/ 2000	M/ 2000 M/ 2000	M/ 2000 M/ 2000	M/ 2000	M/ 2000	M/ 2000 M/ 2000	M/ 2000 M/ 2000
F/539	2:2'Dipiridyl	— Cu^{2+} Fe^{2+} Zn^{2+} Co^{2+}	M/ 5000	M/ 5000	M/ 5000 — — — —	M/ 5000 — — — —	M/ 5000	M/ 5000	M/ 5000 — — — —	M/ 5000 — — — —
F/540	1:10-Phenanthrolin hydrochlorid	— Cu^{2+} Fe^{2+} Zn^{2+} Co^{2+}	M/20 000	M/20 000	M/20 000 — — — —	M/20 000 — — — —	M/20 000	M/20 000	M/10 000 — — — —	M/10 000 — — — —

Siehe Zeichenerklärung der Tabelle 4.

Vol. 7, 1961

TABELLE 7. DIE FUNGIZIDE WIRKUNG CHELATE-KOMPLEX-BILDENDER VERBINDUNGEN AUF FLÜSSIGEM MAISCHE-NÄHRBODEN

(Einwirkungsdauer: 5 Tage.)

Nr.	Verbindungen	Die fungizid wirkende molare Grenzkonzentration der Verbindung	
		Penicillium simplicissimum	*Saccharomyces cerevisiae*
F/445	8-Hydroxy-chinolin	<M/ 1000	<M/ 1000
F/449	5:7-Dichlor-8-hydroxy-chinolin	M/ 2000	M/ 1000
F/450	5:7-Dibrom-8-hydroxy-chinolin	<M/ 1000	<M/ 1000
F/451	5:7-Dijod-8-hydroxy-chinolin	<M/ 1000	<M/ 1000
F/452	5-Chlor-7-jod-8-hydroxy-chinolin	M/ 2000	M/ 2000
F/454	5:7-Dinitro-8-hydroxy-chinolin	M/10 000	M/ 5000
F/495	1-Nitroso-2-naphtylamin	M/ 2000	M/ 2000
F/508	1-Nitroso-2-naphtol	M/ 2000	M/ 2000
F/509	2-Nitroso-1-naphtol	M/ 2000	M/ 2000
F/511	9-Nitroso-10-phenanthrol	M/ 2000	M/ 2000
F/536	1:2-Naphtochinon-dioxym	M/ 2000	M/ 2000
F/539	2:2'-Dipiridyl	<M/ 1000	<M/ 1000
F/540	1:10-Phenanthrolin hydrochlorid	<M/ 1000	<M/ 1000

Nach Durchschau der Ergebnisse der Tabellen 4.–8. lässt folgendes feststellen:

(1) Durch die Fe^{2+}-Ione wird die fungistatische Wirkung eines jeden Gliedes der von uns betreffs ihres Wirkungsmechanismus untersuchten 8-Hydroxy-chinolin-Derivate auf flüssigem serumlosem Nährboden gänzlich abgewehrt; die Cu^{2+}-Ione steigern die fungistatische Wirkung des 8-Hydroxy-chinolins um das Fünffache des Ursprünglichen und sie wehren diejenige der übrigen untersuchten 8-Hydroxy-chinolin-Derivate gänzlich oder beinahe gänzlich ab.

Die fungistatische Wirkung einiger 8-Hydroxy-chinolin-Derivate wird durch Co^{2+}-Ione gänzlich abgewehrt, diejenige anderer aber wesentlich vermindert. Ihre fungistatische Wirkung wird im allgemeinen durch die MoO_4^{2-}-Ione vermindert, durch die Zn^{2+} und Mn^{2+}-Ione dagegen nicht beeinflusst.

Die fugistatische Wirkung der untersuchten Nitroso-phenole wird durch die Fe^{2+}-Ione abgewehrt, durch die bei den Versuchen angewandten übrigen Schwermetall-Ione dagegen nicht beeinflusst.

Die fungistatische Wirkung des 1-Nitroso-2-naphtylamins (Nr. F/495) und 1:2-Naphtochinon-dioxyms (Nr. F/536) wird durch Fe^{2+}-ione abgewehrt.

Die Wirkung des früheren (Nr. F/495) wehren auch die Co^{2+}-Ione ab, diejenige des letzteren (Nr. F/536) dagegen nur vermindern. Die fungistatische Wirkung des 1-Nitroso-2-naphtylamins (Nr. F/495) wird durch Cu^{2+}, Zn^{2+} und Mn^{2+}-Ione nicht beeinflusst; die des 1:2-Naphtochinon-dioxyms (Nr. F/536) wird durch Cu^{2+} vermindert, durch Zn^{2+} und Mn^{2+}-Ione dagegen nicht beeinflusst.

Die fungistatische Wirkung des 2:2'-Dipiridyls (Nr. F/539) und des 1:10-Phenanthrolin-hydrochlorids (Nr. F/540) wird durch Mn^{2+} und MoO_4^{2-}-Ione nicht beeinflusst, durch Cu^{2+}, Fe^{2+}, Zn^{2+}, und Co^{2+}-Ione aber abgewehrt.

(2) Auf festem Nährboden wehren die Fe^{2+} und Co^{2+}-Ione die fungistatische Wirkung eines jeden von diesen ab; gleicherweise wirken auch die Cu^{2+}-Ione, mit Ausnahme die des 8-Hydroxy-chinolins.

TABELLE 8. DIE DURCH REDUZIERENDE STOFFE VERURSACHTE BEEINFLUSSUNG DER AUF FESTEM MAISCHE-AGAR NÄHRBODEN AUSGEÜBTEN FUNGISTATISCHEN WIRKUNG VON CHELATE-KOMPLEX-BILDENDEN UND AUCH ZUR CHELATE-KOMPLEX-BILDUNG UNFÄHIGEN VERBINDUNGEN

Nr.	Verbindungen	*Penicillium simplicissimum*				*Saccharomyces cerevisiae*			
		(ohne Zugaben)	0,3% Cystein hydrochlorid	0,3% Na-Thioglycolat	0,3% Ascorbinsäure	(ohne Zugaben)	0,3% Cystein hydrochlorid	0,3% Na-Thioglycolat	0,3% Ascorbinsäure
F/445	8-Hydroxy-chinolin	42	30	40	42	30	18	27	—
F/449	5:7-Dichlor-8-hydroxy-chinolin	20	17	20	18	25	20	20	—
F/450	5:7-Dibrom-8-hydroxy-chinolin	14	10	14	10	12	12	12	—
F/452	5-Chlor-7-jod-8-hydroxy-chinolin	15	10	15	12	18	15	15	—
F/454	5:7-Dinitro-8-hydroxy-chinolin	30	23	28	15	30	25	30	16
F/508	1-Nitroso-2-naphtol	20	19	20	—	20	18	20	12
F/509	2-Nitroso-1-naphtol	18	18	17	12	16	16	16	15
F/511	9-Nitroso-10-phenanthrol	12	10	12	10	15	12	14	11
F/536	1:2-Naphtochinon-dioxym	15	15	14	12	15	14	14	12
F/539	2:2′-Dipiridyl	18	15	16	18	22	20	22	20
F/540	1:10-Phenanthrolin hydrochlorid	35	25	30	29	26	20	26	25
F/492	4-Nitroso-dimethylanilin	45	—	33	—	35	15	25	—
F/493	4-Nitroso-diaethylanilin	45	—	30	—	36	10	22	—
F/517	3:5-Dimethyl-4-nitroso-pyrazol	22	12	20	—	10	—	—	—
F/518	1-Phenyl-3:5-dimethyl-4-nitroso-pyrazol	50	15	50	—	32	20	32	—

Siehe Zeichenerklärung der Tabelle 3.

Die fungistatische Wirkung des 8-Hydroxy-chinolins wird zwar durch die Cu^{2+}-Ione auf festem Nährboden nicht völlig abgewehrt, doch beträchtlich vermindert.

Die Zn^{2+} und MoO_4^{2-}-Ione vermögen auf festem Nährboden die fungistatische Wirkung des 8-Hydroxy-chinolins (Nr. F/445), des 5:7-Dinitro-8-hydroxy-chinolins (Nr. F/454), des 2:2'-Dipiridyls (Nr. F/539) und des 1:10-Phenanthrolin-hydrochlorids (Nr. F/540) abzuwehren und diejenige des 5-Chlor-7-jod-8-hydroxy-chinolins (Nr. F/452) zu vermindern.

MoO_4^{2-}-Ione können die fungistatische Wirkung der untersuchten Chelate-Komplex-bildenden Verbindungen—mit Ausnahme die der 2-Nitroso-phenol-Derivate (Nr. F/508, 509, 511) und des 1:2-Naphtochinon-dioxyms (Nr. F/536)—auf festem Nährboden gänzlich abwehren; die Mn^{2+}-Ione sind dagegen nicht imstande auch eine der untersuchten Chelate-Komplex-bildenden Verbindungen signifikant zu beeinflussen.

(3) Die Gegenwart von 10% Rinderserum im flüssigen und festen Nährboden kann die fungistatische Wirksamkeit der untersuchten Chelate-Komplex-bildenden Verbindungen nicht beeinflussen (in Gegensatz zu derjenigen zahlreicher Phenol-Derivate; -s. Teil I unserer Mitteilungsserie), weder auf die Beeinflussbarkeit ihrer fungistatischen Wirkung mittels Schwermetall-Ione einwirken.

(4) Die Resultate unserer "*Aufhebungsversuche*" beweisen, dass die auf flüssigem Nährboden ausgeübte fungistatische Wirkung des 8-Hydroxy-chinolins (Nr. F/445) durch Fe^{2+} und MoO_4^{2-}-Ione innerhalb gewisser Konzentrationsgrenzen bedeutend aufgehoben werden kann; Co^{2+}-Ione können dessen fungistatischen Einfluss auf *Saccharomyces cerevisiae* etwas vermindern, denjenigen auf *Penicillium simplicissimum* jedoch nicht beeinträchtigen.

Die fungistatische Wirkung der betreffs des Wirkungsmechanimus von uns untersuchten Derivate des 8-Hydroxy-chinolins wird durch die Cu^{2+}-Ione in einer gegenüber ihrer fungizid wirkenden Konzentration geringeren Verdünnung völlig aufgehoben, durch die Fe^{2+}-Ione gleichfalls gänzlich oder beinahe; durch die Zn^{2+}, Co^{2+} und MoO_4^{2-}-Ione werden sie jedoch entweder gar nicht (oder nur in geringem, unbedeutendem Masse) beeinflusst.

Die untersuchten Chelate-Komplex-bildenden Nitroso-Verbindungen (Nr. F/495, 508, 509, 511) und das 1:2-Naphtochinon-dioxym (Nr. F/536) erweisen sich in ihrer fungistatisch wirkenden Grenzkonzentration zugleich auch als fungizid wirksam, so vermögen Schwermetall-Ione schon von vornherein nicht deren Wirkung aufzuheben, was auch durch unsere mit Fe^{2+}-Ionen ausgeführten "*Aufhebungsversuche*" bestätigt wird.

Die fungistatische Wirkung des 2:2'-Dipiridyls (Nr. F/539) und des 1:10-Phenanthrolin-hydrochlorids wird durch Cu^{2+}, Fe^{2+}, Zn^{2+} und Co^{2+}-Ione gleicherweise gänzlich aufgehoben.

Die Ergebnisse der "*Aufhebungsversuche*" erweisen sich als gänzlich identisch sowohl auf serumfreiem also auch auf 10% Rinderserum enthaltendem flüssigem Maische-Agar-Nährboden.

(5) Das 8-Hydroxy-chinolin (Nr. F/445) und dessen 5:7-Dibrom und 5:7-Dijod-Derivate (Nr. F/450 und F/451) üben auf *Penicillium simplicissimum* und *Saccharomyces cerevisiae* während 5 Tage Einwirkungszeit noch in der Konzentration von M/1000 keine fungizide Wirkung aus; dessen 5:7-Dichlor- (Nr. F/449) und

5-Chlor-7-jod-Derivate (Nr. F/452) üben dagegen auf beide Mikroorganismen-Arten eine fungizide Wirkung aus.

Das 5:7-Dinitro-8-hydroxy-chinolin (Nr. F/454) ist unter den angewandten Versuchsumständen auf das *Penicillium simplicissimum* in der Konzentration von M/10 000, auf das *Saccharomyces cerevisiae* aber in M/5000 fungizid wirkend.

Die fungizide Grenzkonzentration der Nitroso-Verbindungen von Nr. F/495, 508, 509, 511 sowie des 1:2-Naphtochinon-dioxyms (Nr. F/536) ist auf beide Mikroorganismus-Stämme gleichfalls M/2000.

Das 2:2'-Dipiridyl (Nr. F/539) und das 1:10-Phenanthrolin-hydrochlorid (Nr. F/540) erwiesen sich selbst in M/1000 Konzentration fungizid wirkungslos.

(6) Die fungistatische Wirkung des 4-Nitroso-dimethyl-anilins und des 4-Nitroso-diaethyl-anilins (Nr. F/492 und 493) wird durch die Zugabe von 0,3% Na-Thioglycolat zum Maische-Agar-Nährboden etwas vermindert; es kann aber keine der von unds betreffs ihres Wirkungsmechanismus untersuchten Verbindungen wesentlich beeinflussen.

Die fungistatische Wirkung des 8-Hydroxy-chinolins (Nr. F/445), des 5:7-Dinitro-8-hydroxy-chinolins (Nr. F/457), des 1:10-Phenanthrolin-hydrochlorids (Nr. F/540) wird durch Cystein-hydrochlorid (gleichfalls in 0,3% Konz.) mittelmässig vermindert; es wehrt vollkommen die fungistatische Wirkung des 4-Nitroso-dimethyl-anilins (Nr. F/492) und des 4-Nitroso-diaethyl-anilins (Nr. F/493) ab, vermindert bedeutend die des 3:5-Dimethyl-4-nitroso-pyrazols (Nr. F/517) und dessen 1-Phenyl-Derivats (Nr. F/518). Es kann jedoch die fungistatische Wirksamkeit der auf Tabelle 8 angeführten anderen Verbindungen signifikant nicht beeinflussen.

Die zum Nährboden in 0,3% Konzentration gegebene Ascorbinsäure vermindert wesentlich die auf das *Penicillium simplicissimum* und auch auf *Saccharomyces cerevisiae* ausgeübte fungistatische Wirkung des 5:7-Dinitro-8-hydroxy-chinolins (Nr. F/454) und des 1-Nitroso-2-naphtols (Nr. F/508) und wehrt diejenige von F/492, 493, 517 und 518 ab.

Die Ascorbinsäure wehrt die auf das *Saccharomyces cerevisiae* ausgeübte fungistatische Wirkung des 8-Hydroxy-chinolins und dessen 5:7-Dichlor-, 5:7-Dibrom-, 5-Chlor-7-jod-Derivate (Nr. F/445, 449, 450, 452) ab, sie kann aber ihre auf *Penicillium simplicissimum* ausgeübte Wirkung wesentlich nicht beeinflussen.

Die Ascorbinsäure vermag die fungistatische Wirkung weder der Verbindungen von Nr. F/509, 511, 536, noch diejenige des 2:2'-Dipiridyls und des 1:10-Phenanthrolin-hydrochlorids (Nr. F/539 und 540) auf irgendeinen Pilzstamm signifikant zu vermindern.

C. BESPRECHUNG

Wenn wir die Resultate überblicken, die wir im Laufe der Untersuchung der Abwehr und Aufhebung der auf *Penicillium simplicissimum* und auf *Saccharomyces cerevisiae* ausgeübten fungistatischen Wirkung der von uns hinsichtlich ihres Wirkungsmechanismus untersuchten Chelate-Komplex-bildenden Verbindungen mittels verschiedener Schwermetall-Ione gewonnen haben, kommen wir zu folgenden Schlussfolgerungen:

(a) Einige Schwermetall-Ione können die fungistatische Wirkung der 8-Hydroxy-chinolin-Derivate nicht nur abwehren, sondern auch aufheben. Ihre fungistatische Wirkung können also einige Schwermetall-Ione nicht nur durch die mit ihnen

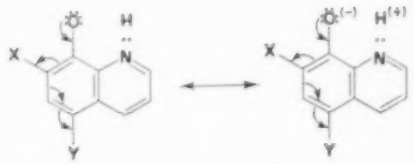
Vol. 7
1961

verbundene extrazellulare Chelate-Komplex-Bildung verhindern, sondern vermögen auch die durch sie intrazellular bewirkte Enzym-Hemmung auch wirklich aufzuheben.

Die in der Abwehr der fungistatischen Wirkung und andererseits in der Aufhebung dieser Wirkung mitwirkenden aktiven Schwermetall-Ione brauchen nicht unbedingt identisch zu sein.

(b) Indem die fungistatische Wirkung des 8-Hydroxy-chinolins nicht genau durch dieselben Schwermetall-Ione abgewehrt bzw. aufgehoben wird—oder nicht unbedingt in demselben Masse—als die Wirkung einiger seiner wirksamen Derivate, deshalb müssen wir voraussetzen, dass deren Angriffspunkt innerhalb der Zellen eines und desselben Pilzstammes je nach den Verbindungen voneinander abweichend sind.

(c) Die fungistatische Wirkung der von uns untersuchten Halogen- und Nitro-Derivate des 8-Hydroxy-chinolins ist wahrscheinlich deshalb intensiver als diejenige des 8-Hydroxy-chinolins selbst, da sie infolge des elektrophylen Charakters der in ihren Molekülen anwesenden Halogen-Atome bzw. Nitro-Gruppen in grösserem Masse als Zwitter-Ione zu dissoziiren vermögen, als das unsubstituierte 8-Hydroxy-chinolin; infolgedessen ist die Intensität ihrer Chelate-Komplex-bildenden Fähigkeit—womit ihre fungistatische Wirkung mehr oder weniger eng zusammenhängt—hochgradiger, als diejenige des unsubstituierten 8-Hydroxy-chinolins. Die Reaktion der Chelate-Komplex-Bildung zwischen den 8-Hydroxy-chinolin-Derivaten und Schwermetall-Ionen ist nach ihrem Wesen ionischer Natur, d.h. es sind eigentlich die Zwitter-Ionformen dieser Verbindungen, die mit den Schwermetall-Ionen in Reaktion treten:
In anschaulicher Form:



X, Y = Halogen-atome, bzw. Nitro-Gruppen

(d) Indem die fungistatische Wirkung der 8-Hydroxy-chinolin-Derivate auf festem Nährboden durch Cystein-hydrochlorid oder Ascorbinsäure im allgemeinen mehr oder weniger vermindert werden kann, deren auf *Saccharomyces cerevisiae* ausgeübter fungistatischer Einfluss sogar—immerhin mit Ausnahme den der Verbindung von Nr. F/454—durch Ascorbinsäure gänzlich aufgehoben wird, so müssen wir voraussetzen, dass der Mechanismus ihrer fungistatischen Wirkung ähnlich sein soll, als derjenige ihrer bakteriostatischen Wirkung: sie bilden nämlich mit einzelnen im Nährboden anwesenden Schwermetall-Ionen Lipoid-lösliche Chelate-Komplexe von der Zusammensetzung 2 mol Agens: 1 mol Schwermetall, welche durch die Zellenwand dringend sich in Komplexe von der Zusammensetzung 1 mol Agens: 1 mol Schwermetall verwandeln, und diese letzteren katalisieren die Fort-Oxidierung der SH-Radikale einiger lebenswichtiger Sulfhydryl-Enzyme der Mikroorganismen (Siehe die Untersuchungen von *Rubbo* und Mitarbeitern[22-23]).

Die reduzierenden Stoffe können vermutlich ihre fungistatische Wirkung dadurch abwehren, dass sie die Fort-Oxidierung der Sulfhydryl-Radikale durch die

Chelate-Komplexe der 8-Hydroxy-chinolin-Derivate verhindern, oder aber dadurch, dass sie die zu —S—S-Gruppen oxidierten (und dadurch inaktivierten) Sulfhydryl-Radikale der Sulfhydryl-Enzyme wieder zu aktiven —SH-Gruppen reduzieren.

Die Möglichkeit der Aufhebung der fungistatischen Wirkung der 8-Hydroxy-chinolin-Derivate durch einige Schwermetall-Ione erklären wir so, dass jene das 8-Hydroxy-chinolin-Derivat-Me_z^+-Komplex—das die Fort-oxidierung der Sulfhydryl-Enzyme katalisiert—intrazellular kompetitive umwandeln in irgendeinen zu dieser katalitischen Reaktion unfähigen 8-Hydroxy-chinolin-Derivat-Me_y^+-Komplex und dadurch die Reduktion der geschädigten Sulfhydryl-Enzyme durch andere Vorgänge der Mikroorganismenzellen ermöglichen, welche Zelle zwar Stoffwechsel ausüben, aber sich nicht vermehren; dadurch wird der weiteren Vermehrung Veranlassung gegeben.

Die Abwehr der fungistatischen Wirkung der 8-Hydroxy-chinolin-Derivate durch einzelne Schwermetall-Ione (z.B. durch Fe^{2+}) erfolgt vermittels eines den früheren gleichen Mechanismus; ihre Abwehr durch andere Ione (z.B durch Co^{2+}) lässt sich aber wahrscheinlich dem Umstand zuschreiben, dass jene mit ihnen extrazellular einen Chelate-Komplex von der Zusammensetzung 2 mol Agens : 1 mol Schwermetall bilden; Die Lösbarkeit dieses Komplexes in dem Nährboden ist aber so niedrigen Grades, dass die Konzentration des betreffenden 8-Hydroxy-chinolin-Derivates (bzw. dessen Schwermetall-Komplexes) in dem Nährboden dadurch unter die Grenzkonzentration fungistatischer Wirkung sinkt.

(e) Der Mechanismus der fungistatischen Wirkung des 2:2′-Dipiridyls (Nr. F/539) und des 1:10-Phenanthrolin-hydrochlorids (Nr. F/540) is ganz andersartig, als derjenige der 8-Hydroxy-chinolin-Derivate. Deren fungistatische Wirkung wird durch reduzierende Stoffe wesentlich nicht vermindert; sie kann durch Cu^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}-Ione gleicherweise gänzlich abgewehrt und auch aufgehoben werden.

Mit Rücksicht darauf müssen wir voraussetzen, dass diese zwei Verbindungen ihre fungistatische Wirkung einfach durch die intrazellulare Chelatierung der Schwermetall-Komponenten gewisser schwermetallhaltiger Enzyme zur Geltung bringen, die zur Vermehrung der Pilz-Zellen unumgänglich notwendig sind. Die betreffenden Enzyme werden dadurch inaktiviert, welche Inaktivität infolge der zum Nährboden im Überschuss zugegebenen Schwermetall-Ione wieder aufgehoben werden kann.

(f) Im fungistatischen Wirkungsmechanismus der Nitroso-Verbindungen von Nr. F/495, 508, 509, 511, sowie des 1:2-Naphtochinon-dioxyms (Nr. F/536) soll vermutlich auf irgendeine Weise auch ihre chelatierende Fähigkeit mitwirken, dies bildet jedoch wahrscheinlich keinen so entscheidenden Faktor für ihren Wirkungsmechanismus, wie für den der 8-Hydroxy-chinolin-Derivate. Der Mechanismus ihrer fungistatischen Wirkung soll auf der *spezifischen und irreversiblen* Hemmung eines bisher noch unbekannten intrazellularen biochemischen Vorganges der Pilz-Zellen beruhen.

(g) Die Abwehr bzw. die Verminderung der fungistatischen Wirkung der zur Chelate-Komplex-Bildung unfähigen Verbindungen von Nr. F/492, 493, 517 und 518 mittels reduzierenden Stoffe können wir damit erklären, dass ihre Nitroso-Gruppe durch die reduzierenden Stoffe extrazellular zu Amino-Gruppen reduziert und demzufolge sie in inaktive Verbindungen verwandelt werden. Daraus ergibt sich zugleich auch die Folge, dass die Nitroso-Gruppe in ihrem Molekül die Vorbedingung für ihre fungistatische Wirksamkeit bildet.

Vol. 7
1961

Wir müssen voraussetzen, dass die fungistatische Wirkung der Verbindungen von Nr. F/492, 493, 517 und 518 durch ihre Nitroso-Gruppe veranlasst wird, welche Wirkung diese Gruppen durch Reagierung mit gewissen reaktiven Radikalen näher noch nicht bekannter, doch zur Vermehrung unumgänglich notwendiger Enzyme der Pilz-Zellen ausüben.

ZUSAMMENFASSUNG

Verfasser hat die fungistatische Wirkung von 41 8-Hydroxy-chinolin-Derivaten, 33 Nitroso-Verbindungen, 20 Oxymen und 10 Phenanthrolin-Derivaten auf einem 10% Rinderserum enthaltenden flüssigen und 3% Agar enthaltenden festen Maische-Nährboden untersucht und zugleich auch den Wirkungsmechanismus derjenigen studiert, die unter diesen am wirksamsten gefunden wurden. Er suchte in der Reihe dieser Verbindungen Zusammenhänge zu finden einerseits zwischen der Chelate-Komplex-bildenden Fähigkeit und der fungistatischen Wirksamkeit, andererseits aber zwischen der Beeinflussbarkeit der fungistatischen Wirkung durch reduzierende Stoffe und dem Wirkungsmechanismus.

Er suchte dabei Schlüsse bezüglich des Wirkungsmechanismus dieser Reihe von Verbindungen zu ziehen.

LITERATUR

1. T. Zsolnai, *Biochem. Pharmacol.* **5**, 1 (1960).
2. T. Zsolnai, *Biochem. Pharmacol.* **5**, 287 (1961).
3. E. Schraufstätter, R. Richter und W. Ditscheid, *Arch. Derm. Syph.* **188**, 259 (1949–50).
4. E. Schraufstätter, *Z. Naturf.* **5b**, 190 (1950).
5. J. Uri und G. Szabó, *Acta Phys. Acad. Sci. Hung.* **3**, 425 (1952).
6. P. Gavandan, *Mém. Serv. Chim. État (Paris)* **32**, 418 (1945); *Chem. Abstr.* p. 5601 (1948).
7. K. A. Oster und M. J. Golden, *J. Amer. Pharm. Ass.* **37**, 429 (1948).
8. W. O. Kermack, *Sci. Progr.* **36**, 283 (1948).
9. W. Jadassohn und E. Pfanner, *Dermatologica* **91**, 46 (1945).
10. W. Jadassohn, E. Pfanner und W. Hausmann, *Schweiz. Med. Wochenschr.* **77**, 987 (1947).
11. R. W. Marsh, *Nature, Lond.* **167**, 97 (1951).
12. M. L. Littman, *Trans. N.Y. Acad. Sci.* **18**, (2), 161 (1955).
13. J. R. Frey, *Dermatologica* **107**, 88 (1953).
14. J. R. Frey, *Dermatologica* **107**, 60 (1953).
15. O. Beck und H. Lacy, *Texas Rep. Biol. Med.* **9**, 395 (1951).
16. S. S. Block, *Agric. Food Chem.* **3**, 229 (1955).
17. C. L. Mason, *Phytopathology* **38**, 740 (1948).
18. K. A. Oster und M. J. Golden, *Exp. Med. Surg.* **1**, 37 (1949).
19. K. Sigg, *Schweiz. Med. Wochenschr.* **77**, 123 (1947).
20. A. Albert, *Med. J. Aust.* **1**, 245 (1944).
21. A. Albert, S. D. Rubbo, R. J. Goldacre und B. G. Balfour, *Brit. J. Exp. Path.* **28**, 69 (1947).
22. S. D. Rubbo, A. Albert und M. I. Gibson, *Brit. J. Exp. Path.* **31**, 425 (1950).
23. A. Albert, M. I. Gibson und S. D. Rubbo, *Brit. J. Exp. Path.* **34**, 119 (1953).
24. A. Albert, A. Hampton, F. R. Selbie und R. D. Simon, *Brit. J. Exp. Path.* **35**, 75 (1954).
25. E. Jeney, T. Zsolnai, Z. Melles und P. Juhász, *Zbl. Bakt.* I. Orig **160**, 677 (1954).
26. E. Jeney, T. Zsolnai und Z. Melles, *Zbl. Bakt.* I. Orig **161**, 465 (1954).
27. E. Jeney und T. Zsolnai, *Zbl. Bakt.* I. Orig **163**, 281 (1955).
28. E. Jeney und T. Zsolnai, *Zbl. Bakt.* II. Orig **110**, 634 (1957).
29. Carl und Marquardt, *Z. Naturf.* **4b**, 280 (1949).
30. M. Vajda und T. Nógrádi, *Experientia* **10**, 373 (1954).
31. E. Sorkin, W. Roth und H. Erlenmeyer, *Experientia* **7**, 64, 257 (1951).
32. W. Roth, E. Sorkin und H. Erlenmeyer, *Schweiz. Z. Allg. Path. Bakt.* **15**, 300 (1952).

33. T. URBANSKI, S. SLOPEK und J. VENULET, *Nature, Lond.* **168**, 29 (1951).
34. A. A. KNIGHT, D. W. TARUN und J. MILLER, *Amer. J. Trop. Med.* **29**, 871 (1949).
35. D. J. TAYLOR und J. GREENBERG, *Amer. J. Hyg.* **56**, 58 (1952).
36. M. M. GREEN, *J. Amer. Pharm. Ass.* **7**, 150 (1946).
37. J. H. ARNETT, *Amer. J. Med. Sci.* **213**, 608 (1947).
38. H. H. ANDERSON, N. A. DAVID und D. A. KOCH, *Proc. Soc. Exp. Biol., N.Y.* **28**, 484 (1931).
39. W. BALAMUTH, *Amer. J. Trop. Med.* **2**, 191 (1953).
40. T. C. MORTON, *Brit. Med. J.* **1**, 831 (1945).
41. W. R. JONES, *Brit. J. Pharmacol.* **2**, 217 (1947).
42. P. E. THOMPSON und B. L. LILLIGREN, *Amer. J. Trop. Med.* **29**, 323 (1949).
43. L. G. GOODWIN, C. A. HOARE und T. M. SHARP, *Brit. J. Pharmacol.* **3**, 44 (1948).
44. P. E. THOMPSON, J. W. REINERTSON, A. BAYLES, D. A. MCCARTHY und E. F. ELSLAGER, *Amer. J. Trop. Med.* **4**, 224 (1955).
45. G. JOHNSON und R. E. TRUSSEL, *Proc. Soc. Exp. Biol., N.Y.* **54**, 245 (1943).
46. R. E. TRUSSEL und G. JOHNSON, *Amer. J. Obstet. Gynec.* **48**, 215 (1944).
47. G. A. ZENTMYER, *Science* **100**, 294 (1944).
48. T. URBANSKI, S. HORNUNG, S. SLOPEK und J. VENULET, *Nature, Lond.* **170**, 753 (1952).
49. A. GOTH, *Science* **104**, 330 (1946).
50. F. BLANK, *Nature* **168**, 516 (1951).
51. H. J. CHINN, R. B. MITCHELL und A. C. ARNOLD, *J. Invest. Derm.* **20**, 177 (1953).

Vol.
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 221-225. Pergamon Press Ltd., Printed in Great Britain.

THE REDUCTION OF CYTOCHROME *C* BY REDUCED VITAMIN K_1*

WALTER D. WOSILAIT

Department of Pharmacology, State University of New York,
Downstate Medical Center, Brooklyn 3, New York

(*Received* 21 *January* 1961)

Abstract—The mitochondrial fraction of rat liver catalyzes the reduction of cytochrome *c* by reduced vitamin K_1. This activity, which has been partially solubilized, is inhibited to only a slight extent by the anticoagulants. The reduction of cytochrome *c* is accompanied by the oxidation of reduced vitamin K_1.

INTRODUCTION

ALTHOUGH previous work with vitamin K has been largely concerned with its role in blood clotting, recent studies have indicated that it may be involved in electron transport. For example, the reduction of menadione (2-methyl-1:4-naphthoquinone) and vitamin K_1 (2-methyl-3-phytyl-1:4-naphthoquinone) by the reduced pyridine nucleotides is catalyzed by an anticoagulant-sensitive enzyme designated pyridine nucleotide-vitamin K_1 reductase.[1, 2] In order to determine whether the reduced form of vitamin K_1 (vitamin K_1H_2) might in turn reduce a cytochrome such as cytochrome *c*, cell fractions of rat liver were tested for such catalytic activity. The mitochondrial fraction catalyzed the reduction of cytochrome *c* by vitamin K_1H_2 and an attempt to dissociate this activity from the mitochondria was partially successful. The present report is concerned with some of the properties of this fraction, which is tentatively designated vitamin K_1H_2–cytochrome *c* reductase.

METHODS

The standard assay system contained the following components: buffer of pH 6·2 containing citrate and ethylenediaminetetra-acetic acid (EDTA) (60 and 6 μmoles, respectively), 10 μmoles of neutralized KCN, 0·087 μmole of vitamin K_1H_2, 0·038 μmoles of horse heart ferri-cytochrome *c* (Sigma Chemical Co), water, and enzyme to a final volume of 1·0 ml. The reactions, which were started by the final addition of enzyme, were measured by following the increase in absorbance at 550 mμ, which is characteristic of the reduction of the alpha band of cytochrome *c*, in a Beckman model DU spectrophotometer at about 25 °C. The increase in absorbance was measured at 30-sec intervals for the first 2 min and at 1-min intervals thereafter for 5 min. Relative activities were based on the net change in absorbance observed over the 5-min period, after corrections were made for the non-enzymic contribution. In most of these studies from 50 to 150 μg of partially purified enzyme were used under conditions in which enzyme was the limiting factor.

* This research was supported, in part, by a grant (H3055) from the U.S. Public Health Service.

Vitamin K_1 was solubilized as described previously[2] and reduced as follows: 4 μmoles of solubilized vitamin K_1 in 12 ml of water were reduced with 200 μmoles of freshly dissolved potassium borohydride (KBH_4). The mixture was allowed to stand for 10 min at room temperature, after which the excess KBH_4 was destroyed by adjusting the pH to 1·9 with 1 N HCl and allowing the mixture to stand for 30 min at 0 °C before use. The reduced samples were kept at 0 °C and were protected from light with aluminium foil during use. Since there was a gradual oxidation of the reduced vitamin, these samples were used for no more than 6 hr. The ultraviolet spectrum of the chemically reduced material was similar to that of enzymically reduced vitamin K_1,[2] and after reoxidation the material migrated in the same manner as vitamin K_1, as shown by paper chromatography. The spectral changes observed after chemical reduction were similar to those observed after enzymic reduction, hence the chemically reduced substance is designated vitamin K_1H_2, but since other changes may have occurred, which were not detectable by spectrophotometric or chromatographic procedures, the designation must be tentative. Solvent controls containing BRIJ-35 (polyoxyethylene lauryl alcohol), without vitamin K_1, were treated in the same way.

RESULTS AND DISCUSSION

In intracellular distribution studies, 85 per cent or more of the vitamin K_1H_2–cytochrome *c* reductase activity of the homogenate was associated with the mitochondrial fraction, while the soluble and microsomal fractions contained little or no activity. As a routine procedure, the enzyme was prepared, by a procedure described elsewhere,[3] from twice-washed rat liver mitochondria which were isolated in 0·25M sucrose containing 10^{-3} M EDTA of pH 7·4. The mitochondria were diluted with sucrose–EDTA to a concentration of about 20 mg of protein per ml and stored at −17 °C until the time of use. After thawing, the samples were centrifuged for 15 min at 15,000 **g**. The collected sediment, which contained the activity, was suspended in sucrose–EDTA and brought to one-fourth the original volume. Partial dissociation of the activity was attained by adding a solution of 10% Lubrol WX (Arnold Hoffman & Co, Inc.) to the sample to make a 2% solution, and the mixture was allowed to stand for 30 min, after which it was centrifuged as before. The sediment was discarded and the supernatant fluid was then centrifuged for 1 hr at 100,000 **g**. The collected sediment, which contained most of the activity, was suspended in one-fourth the previous volume of sucrose–EDTA and diluted with an equal volume of 0·1 M imidazole buffer pH 7·4 for storage at −17 °C. Such preparations were used for most of these studies.

The time course of reduction of cytochrome *c* in a typical reaction is illustrated in Fig. 1 by the increase in absorbance at 550 mμ. Cyanide was required in the system for reduction to be observed, presumably because of the presence of cytochrome oxidase in the preparation. Similar results were obtained under anaerobic conditions. Heating the non-dialysable reductase for 10 min at 100 °C resulted in a loss of 80 per cent or more of its activity. Other proteins, such as bovine serum albumin, crystalline yeast hexokinase, phosvitin, and ovalbumin at similar concentrations, did not appear to catalyze this reaction. Chemically reduced menadione also served as an electron donor in this system; however, the rate of non-enzymic reduction of cytochrome *c* by reduced menadione was greater than with reduced vitamin K_1. Hydroquinone also served in place of vitamin K_1H_2; reduced coenzyme Q_{10} has not yet been tried.

Vol. 1
1961

Definitive specificity studies will be carried out when more pure enzyme preparations become available.

The oxidation of vitamin K_1H_2, during the reduction of cytochrome *c*, was followed by observing the spectral changes in the 250 to 290 mμ region of the ultraviolet, where the absorbance by the oxidized and reduced forms of the vitamin are different. However, to follow such changes it was necessary to modify the standard assay system to suboptimal conditions which resulted in a greater non-enzymic contribution to the

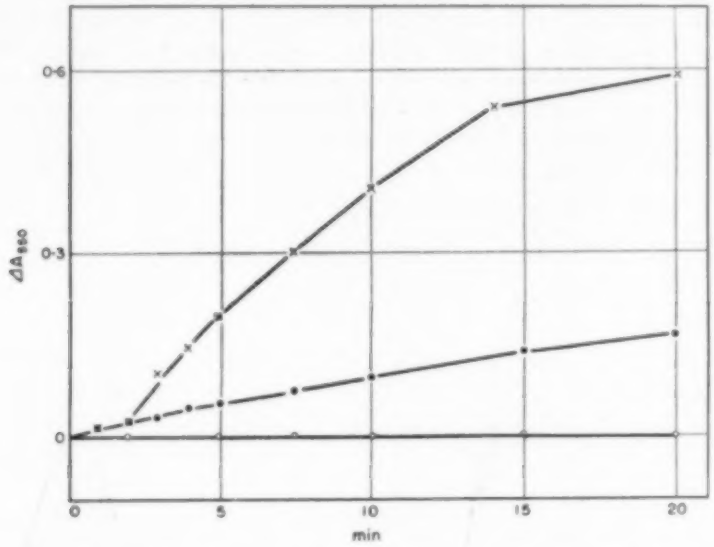


FIG. 1. Reduction of cytochrome *c* by vitamin K_1H_2. The standard assay was carried out as described in the text. At 2 min, 270 μg of enzyme was added to the complete system (× —— ×), and also to the control, in which BRIJ-35 without vitamin K_1 was used (○——○). Enzyme was omitted from sample (●——●) in order to follow the non-enzymic reduction of cytochrome *c*.

overall reaction. As illustrated in Fig. 2, the spectrum between 240 and 290 mμ changed from one typical of vitamin K_1H_2 (0 min) to one typical of vitamin K_1 (20 min) during the reduction of cytochrome *c*. Moreover, there was an increase in absorbancy between 290 and 340 mμ which may be related to the reduction of cytochrome *c*; however, there is some uncertainty concerning this point.[4] It may be pointed out that when beef heart and different horse heart cytochrome *c* preparations were used, similar spectral changes were observed. The spectral changes which occurred in the absence of enzyme were similar, but the rate was much reduced. The non-enzymic contribution in the experiment illustrated was greater than usual, for reasons given above, and in this experiment this contribution was estimated to be about 40 per cent of the overall change. A comparison of the changes observed at 272 mμ with those observed at 550 mμ showed about a 1:1 relationship between the amount of vitamin K_1H_2 oxidized and the amount of cytochrome *c* reduced. This is greater than expected, and might be due, in part, to the fact that these reactions were carried out under aerobic conditions. Experiments are in progress to clarify this point.

The enzyme was inhibited about 50 per cent by bishydroxycoumarin (Dicumarol), acenocoumarol (Sintrom), cumachlor, and phenindione, at a concentration of 10^{-4} M, and about 20 per cent by ethyl biscoumacetate (Tromexan) and warfarin at the same concentration. The possibility that Dicumarol might accept electrons from vitamin K_1H_2 and compete with cytochrome *c*, thus producing an apparent inhibition, was considered, but on the basis of spectral studies carried out, this did not appear to take place under the standard assay conditions. In general, vitamin K_1H_2–cytochrome *c* reductase was less sensitive to the anticoagulants than was the pyridine nucleotide–vitamin K_1 reductase.[2] Antimycin A caused about 85 per cent inhibition at a concentration of 0·1 μg per ml. 2:4-Dinitrophenol caused about 40 per cent inhibition at 10^{-4} M, while *p*-nitrophenol and 2:4-dinitroaniline had little or no effect. Metal binders such as α:α'-dipyridyl, orthophenanthroline and EDTA, at concentrations of 10^{-4} M, did not appear to influence the activity.

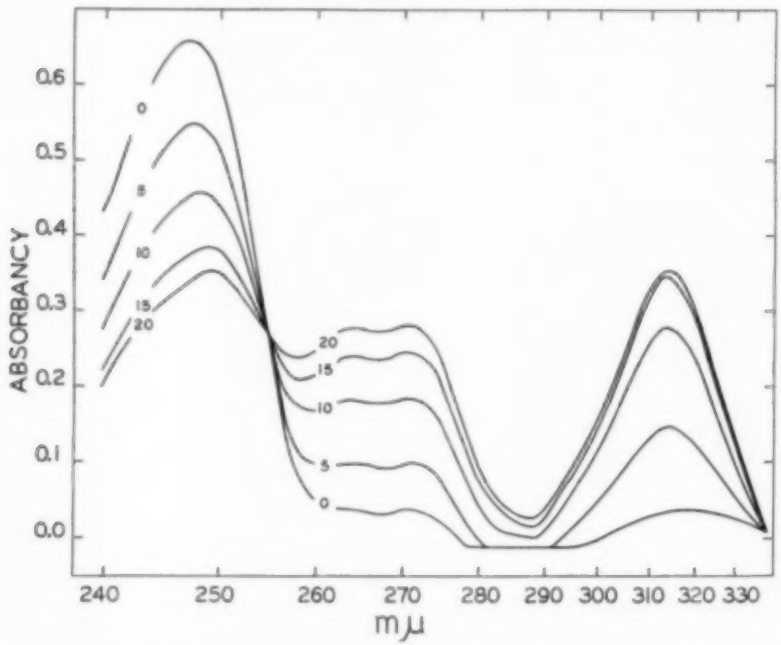


FIG. 2. Oxidation of vitamin K_1H_2 during reduction of cytochrome *c*. The standard assay system was increased to 3·0 ml and the concentration of vitamin K_1H_2 was reduced to 0·017 μmole per ml; thus, the spectral changes which took place could be followed in a Beckman model DK2 recording spectrophotometer.* The reference cell contained all components, including enzyme, except vitamin K_1H_2. The 0-min recording was started immediately after enzyme addition, while the others were started at the indicated times; hence, these changes indicate the general course of the reaction.

* Generously made available by Dr E. Muntwyler.

Although vitamin K_1 has not been unequivocally identified in mammalian tissue, Green and Dam reported that the mitochondrial fraction of beef liver contained a factor which behaved as vitamin K in the bioassay.[5] In addition, Martius reported that ^{14}C-labeled menadione was converted to a substance which was associated with

Vol. 7
1961

the mitochondria,[6] and the product was later reported to be 2-methyl-3-(geranyl-geranyl)-1:4-naphthoquinone in chicken and rat.[7] Another quinone, coenzyme Q, is also associated with the mitochondria, but, according to Lester *et al.*, members of this series did not elevate the prothrombin levels of vitamin K-deficient chicks; similarly, coenzyme Q_{10} did not restore the prothrombin level of rats treated with warfarin.[8] Since the present reaction has been studied with a particle which is undoubtedly complex, the possibility that vitamin K_1H_2 may be non-enzymatically reducing a component, such as vitamin K or coenzyme Q, or even some other unidentified factor associated with the particle, and in this fashion introducing electrons into the electron transport chain, cannot be excluded. Since the details of this reaction are not yet known and the reduction of cytochrome *c* is inhibited by Antimycin A, it is possible that a system similar to that described in heart by Ambe and Crane,[9] which involves coenzyme Q and is sensitive to Antimycin, may be present in the preparations used and involved in this reaction. As yet, neither the yield nor the purity of the preparations has been great enough to permit satisfactory analyses for substances such as vitamin K or coenzyme Q. Although complete solubilization of the factor in rat liver has not been attained, fractions were found in dog liver and spinach which did not sediment at 100,000 **g** yet catalyzed the reduction of cytochrome *c* by reduced vitamin K_1. The view that vitamin K may be involved in electron transport is supported by the results of previous studies, in which the reduction of vitamin K_1 was described,[2] and also by the results of the present investigation, in which the subsequent transfer of electrons to cytochrome *c* is described. Further studies are in progress in order to clarify the nature of these reactions and to determine the identity of the natural electron carrier, in order that the possible role of vitamin K in electron transport can be more accurately evaluated.

REFERENCES

1. W. D. Wosilait and A. Nason, *J. Biol. Chem.* **208**, 785 (1954).
2. W. D. Wosilait, *J. Biol. Chem.* **235**, 1196 (1960).
3. W. C. Schneider and G. H. Hogeboom, *J. Biol. Chem.* **183**, 123 (1950).
4. R. Lemberg, *Hematin Compounds and Bile Pigments*, p. 349. Interscience, New York (1949).
5. J. P. Green, E. Søndergaard and H. Dam, *Biochim. et Biophys. Acta* **19**, 182 (1956).
6. C. Martius, *Biochem. Z.* **327**, 407 (1956).
7. C. Martius and O. H. Esser, *Biochem. Z.* **331**, 1 (1958).
8. R. L. Lester, Y. Hatefi, C. Widmer and F. L. Crane, *Biochim. et Biophys. Acta* **33**, 169 (1959).
9. K. S. Ambe and F. L. Crane, *Biochim et Biophys. Acta* **43**, 30 (1960).

Biochemical Pharmacology, 1961, Vol. 7, pp. 226–231. Pergamon Press Ltd., Printed in Great Britain.

METABOLIC STUDIES ON N-METHYLPYRIDINIUM-2-ALDOXIME—I

THE CONVERSION TO THIOCYANATE

INGER ENANDER, ANDERS SUNDWALL AND BO SÖRBO

Research Institute of National Defence, Dept. 1, Sundbyberg and Nobel Medical Institute, Biochemical Dept., Stockholm, Sweden

(*Received* 15 *March* 1961)

Abstract—N-methylpyridinium-2-aldoxime methanesulphonate (P2S) was administered to rats and the urinary excretion of thiocyanate was determined by different methods. In addition to a small increase in thiocyanate excretion a metabolite was found which interfered with one of the test methods. It was concluded that formation of cyanide could not account for the toxicity of the oxime. Similar experiments on human beings showed no significant increase in thiocyanate excretion, but small amounts of an unknown metabolite were formed.

Vol. 7
1961

THE value of certain oximes as antidotes against nerve gases or organophosphorus insecticides is well established.[1, 2] One of the most efficient of these oximes is N-methylpyridinium-2-aldoxime, used either as the iodide (PAM) or the more soluble methanesulphonate (P2S). Although little is known about the metabolic fate of this oxime, conversion of some oximes to cyanide (for instance di*iso*nitrosoacetone (DINA)) has been demonstrated and is presumably responsible for their toxicity.[3] Similar experiments have apparently not been carried out on N-methylpyridinium-2-aldoxime but it is known that this compound slowly decomposes to cyanide at neutral pH.[4] Cyanide and P2S poisoning have certain features in common, i.e. they show the same symptoms (respiratory stimulation followed by arrest and convulsions) and the dog is the most sensitive animal for both.[5, 6] It has also been observed in this laboratory (unpublished) that a combination of sodium nitrite and sodium thiosulphate, a well-known antidote combination against cyanide, decreases the toxicity of N-methylpyridinium-2-aldoxime, indicating that formation of cyanide could contribute to the toxicity of the oxime.

To test conversion of N-methylpyridinium-2-aldoxime to cyanide in the body, the urinary excretion of thiocyanate was followed after administration of the oxime. This technique is based on the fact that cyanide is rapidly converted *in vivo* to thiocyanate by enzymatic processes.[7, 8]

MATERIALS AND METHODS

N-methylpyridinium-2-aldoxime methanesulphonate (P2S) was prepared according to Creasy and Green.[9] *Di*isonitrosoacetone (DINA) was prepared according to Koessler and Hanke.[10] All other compounds used were commercial products of analytical purity.

Rats of either sex weighing 200–300 g were kept in metabolism cages and fed a diet of low thiocyanate content.[11] The human volunteers were male students (non-smokers) who were allowed to eat a free diet. Thiocyanate analysis was carried out by three different methods. *Method I* was a modification[12] of the procedure of Boxer and Richards[13] based upon oxidation of the thiocyanate to hydrogen cyanide by permanganate in acid solution. The hydrogen cyanide is transfered into sodium hydroxide and cyanide is then determined colorimetrically by Zincke–Königs reaction. Although this method is claimed to be specific for thiocyanate, experiments described in the following section demonstrated that a metabolite from P2S gave a positive interference in this method. *Method II* was developed from the observation that the procedure could be made more specific by a preliminary precipitation of thiocyanate with silver ions. The difference between the value obtained by method I applied directly to the sample and that obtained after silver precipitation was then taken to represent the thiocyanate content of the sample. Cyanide (hydrocyanic acid) would interfere in this method, but it was verified (by acidification and aeration of the samples as in method I, but with the oxidation step omitted) that the urine did not contain any cyanide.

It was also verified that neither metabolites from P2S nor the oxime itself interfered in the silver precipitation step. ^{14}C-labelled thiocyanate, when added to urine samples obtained from P2S-treated animals, was completely removed from the urine by this treatment. The silver precipitation step was carried out as follows: 5 ml of urine (acidified with acetic acid) and 0·9 ml 1 M $AgNO_3$ were mixed and the precipitate removed by centrifugation. To 5 ml of the supernatant was added 1·0 ml 1 M NaCl and the silver chloride removed by centrifugation. *Method III*[14] was based on the red colour given by ferric ions with thiocyanate. This method is considerably less specific for thiocyanate as certain urinary pigments also give coloured products with ferric ions.

RESULTS

In the first part of the investigation the urinary excretion of thiocyanate after intramuscular and oral administration of P2S was determined in rats. When thiocyanate was determined with method I the results shown in Figs. 1 and 2 were obtained. A large increase in thiocyanate excretion was observed, amounting to about 5 and 3 per cent, respectively, of the P2S administered, calculated on an equimolar conversion of P2S to the thiocyanate. However, when the analysis was made by other methods the increase in thiocyanate was much less (Table 1). This indicated the formation of a compound easily converted to cyanide but not identical with thiocyanate. The increase in true thiocyanate (as determined by method II) after P2S administration is shown in Fig. 3. It amounted to about 1 per cent of the P2S injected. It was verified that when P2S was incubated with rat urine for 24 hr neither a formation of thiocyanate nor of the unknown metabolite could be detected.

Attempts to purify the unknown cyanide-forming metabolite (metabolite X) were undertaken. It was observed that it was not adsorbed on the hydrogen form of strong cation exchangers but was completely removed by treatment of solutions with strong anion exchangers (acetate and hydroxide form). However, the metabolite could not

be eluted from the latter. It was possible to adsorb the metabolite on active carbon and a partial elution (to about 30 per cent) could be accomplished with aqueous pyridine. The possibility was considered that metabolite X was 2-imino-4-thiazolidine carboxylic acid (a detoxification product of cyanide),[15] but tests with an authentic specimen of this substance showed that it was not oxidized to cyanide under the test conditions used in method I.

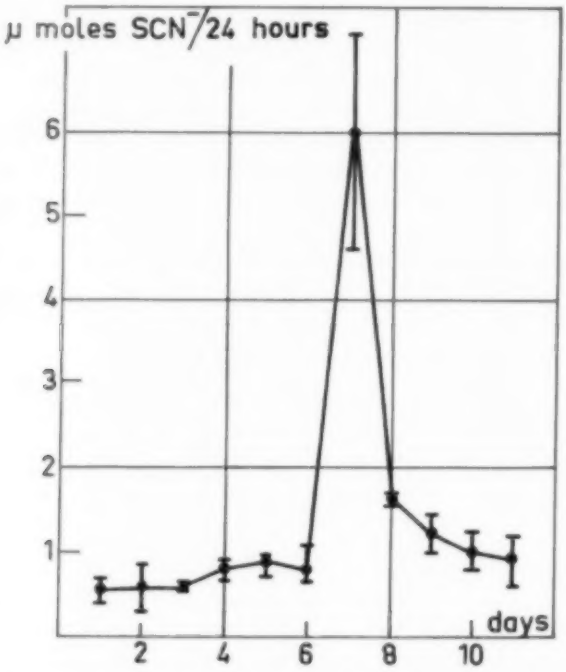


FIG. 1. Thiocyanate excretion (according to method I) after intramuscular injection of P2S. Five rats were injected with P2S (100 mg/kg) on day 6. The dots indicate means and the bars extreme values.

The possibility that metabolite X could be formed from other oximes was also considered. Rats were injected with DINA and their urine analysed as above. About 60 per cent of the injected dose (10 mg/kg body weight intramuscularly, twice a day) was excreted as thiocyanate but no formation of metabolite X could be detected.

To determine whether metabolite X was formed from cyanide, rats were given a sublethal dose of KCN (1 mg KCN per kg body weight intramuscularly, twice a day) and the following 24-hr urine analysed for thiocyanate and metabolite X. No formation of the latter could be detected, but about 50 per cent of the cyanide was excreted as thiocyanate in confirmation of earlier reports in the literature.[16]

Vol. 7
1961

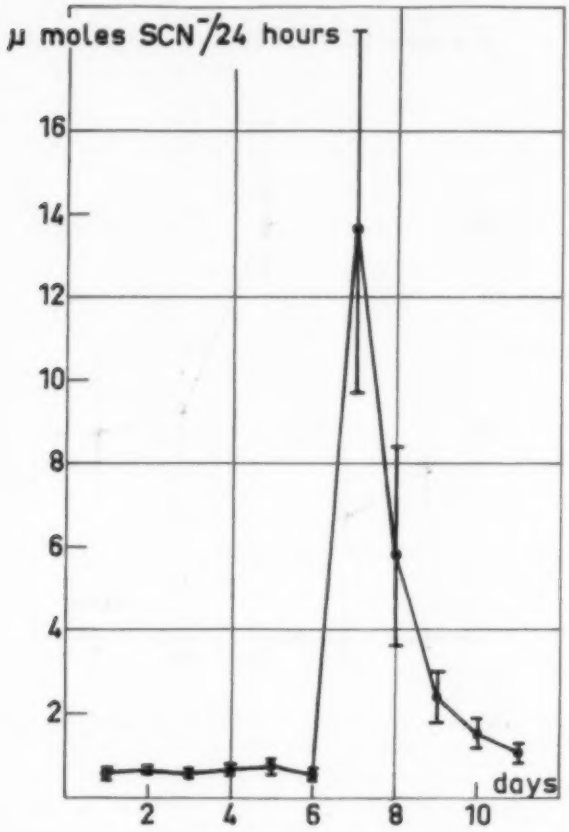


FIG. 2. Thiocyanate excretion (according to method I) after oral administration of P2S. Five rats received 500 mg/kg of P2S on day 6. Other conditions as in Fig. 1.

TABLE 1. THIOCYANATE DETERMINATION ON URINE FROM RATS BEFORE AND AFTER TREATMENT WITH P2S

A rat received 120 µmoles P2S i.m. (corresponding to 100 mg/kg). The 24 hr urine was collected and assayed for thiocyanate by the different methods described in text.

Method	µMoles SCN^-/24 hr	
	Control	After P2S
I	0·85	4·69
II	0·58	0·82
III	5·0	5·2

In the second part of the investigation P2S was administered to human volunteers and the excretion of true thiocyanate and the metabolite X were determined. The results summarized in Table 2, show no significant increase in thiocyanate excretion but a small formation of the unknown metabolite.

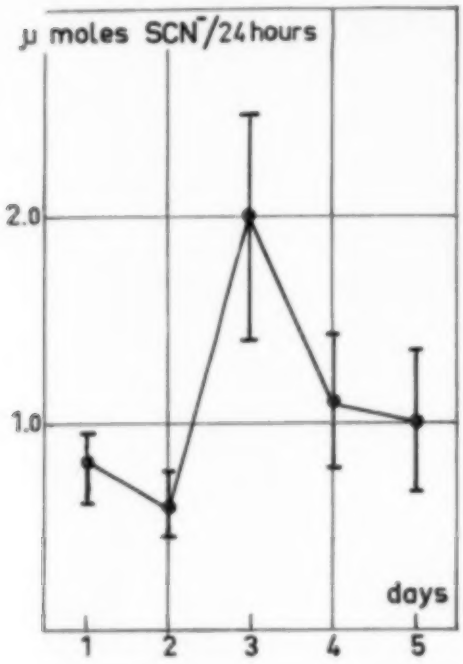


FIG. 3. Thiocyanate excretion (according to method II) after intramuscular injection of P2S. Conditions as in Fig. 1. but P2S was given on day 2.

TABLE 2. THIOCYANATE DETERMINATIONS ON URINE FROM NORMAL AND P2S TREATED HUMAN VOLUNTEERS

The 24-hr urine from four male students was collected and analysed for SCN^- before and after P2S administration (3 g orally in gelatin capsules). The amount of metabolite X excreted was obtained from the difference between the values given by method I and method II. Numbers in brackets indicate extreme values.

Day before treatment	μMoles SCN^-/24 hr		
	Method I	Method II	Metabolite X
1	67·5 (57·0–84·5)	62·7 (52·1–80·0)	4·8 (4·2–5·5)
2	75·7 (49·3–102)	70·8 (43·3–97·0)	4·9 (4·0–6·0)
3	78·9 (47·2–150)	73·3 (43·5–141)	5·6 (2·8–9·0)
Day after treatment			
1	123 (89·5–183)	87·6 (61·1–139)	35 (28·4–44)
2	92·6 (65·2–133)	79·4 (58·5–117)	13·2 (6·0–16·2)
3	73·8 (52·7–113)	66·6 (45·8–108)	7·2 (6·2–9·3)
4	71·8 (54·1–115)	65·5 (48·2–108)	6·3 (5·6–7·4)

DISCUSSION

The present investigation has shown that very little thiocyanate is formed from P2S in rats. The increase in thiocyanate excretion found corresponded to 0·1 mg hydrogen cyanide per kg body weight when 0·5 LD_{50} of P2S was administered.[5] It is reasonable to assume that if one LD_{50} dose of P2S had been given not more than twice as much cyanide (0·2 mg/kg) could be formed. The LD_{50} of cyanide (HCN) after slow poisoning (intraperitoneal injection) is 3·1 mg/kg in rats.[17] Consequently the amounts of cyanide formed from P2S cannot account for the toxicity of the latter.

The increase in thiocyanate (plus metabolite X) excretion found by method I in human beings after P2S *per os* was about 50 μmoles (the increase was in fact not statistically significant) which is equivalent to a 0·4 per cent conversion. The corresponding figure for rat is 3 per cent. Consequently P2S is more extensively metabolized to thiocyanate in rats than in human beings.

Acknowledgement—Grateful appreciation is expressed to Mr. G. Engström and I. Hedenborg for their able technical assistance.

REFERENCES

1. D. R. Davies and A. L. Green, *Brit. J. Ind. Med.* **16**, 128 (1959).
2. J. H. Wills, *Fed. Proc.* **18**, 1020 (1959).
3. B. M. Askew, D. R. Davies, A. L. Green and R. Holmes, *Brit. J. Pharmacol.* **11**, 424 (1956)
4. M. Halse and O. Skogan, *Medd. Norsk Farm. Selskap* **11**, 141 (1959).
5. D. R. Davies and G. L. Willey, *Brit. J. Pharmacol.* **13**, 202 (1958).
6. J. Barcroft, *J. Hyg., Camb.* **31**, 1 (1931).
7. K. Lang, *Biochem. Z.* **259**, 243 (1933).
8. B. Sörbo, *Acta Chem. Scand.* **8**, 694 (1954).
9. H. N. Creasy and A. L. Green, *J. Pharm., London* **11**, 485 (1959).
10. K. Koessler and M. Hanke, *J. Amer. Chem. Soc.* **40**, 1717 (1918).
11. Fromageot and A. Royer, *Enzymologia* **11**, 485 (1943).
12. C. Nyström and B. Sörbo, *Scand. J. Clin. Lab. Invest.* **9**, 223 (1957).
13. G. E. Boxer and J. C. Richards, *Arch. Biochem. Biophys.* **39**, 292 (1952).
14. R. G. Bowler, *Biochem. J.* **38**, 385 (1944).
15. J. L. Wood and S. L. Cooley, *J. Biol. Chem.* **218**, 449 (1956).
16. K. F. Støa. Thesis. Universitetet i Bergen, Årbok (1957).
17. C.-J. Clemedson, T. Fredriksson, B. Hansen, H. Hultman and B. Sörbo, *Acta Physiol. Scand.* **42**, 41 (1958).

Biochemical Pharmacology, 1961, Vol. 7, pp. 232–236. Pergamon Press Ltd., Printed in Great Britain.

METABOLIC STUDIES ON N-METHYLPYRIDINIUM-2-ALDOXIME—II

THE CONVERSION TO N-METHYLPYRIDINIUM-2-NITRILE

INGER ENANDER, ANDERS SUNDWALL AND BO SÖRBO

Research Institute of National Defence, Dept. 1, Sundbyberg and Nobel Medical Institute, Biochemical Dept., Stockholm, Sweden

(*Received* 15 *March* 1961)

Abstract—N-methylpyridinium-2-nitrile has been identified in urine from rats and human beings treated with N-methylpyridinium-2-aldoxime. When injected into rats the nitrile is partially converted to inorganic thiocyanate and a metabolite possibly identical with metabolite X found in urine from oxime-treated animals. The nitrile was somewhat more toxic than the oxime.

EVIDENCE has been reported in the literature[1] for the *in vitro* conversion of N-methylpyridinium-2-aldoxime to N-methylpyridinium-2-nitrile. The aim of the present investigation was to see if the same reaction occurs *in vivo*. An analytical procedure for N-methylpyridinium-2-nitrile (MPN) in biological material has been developed and applied to urine from rats and human beings which had received N-methylpyridinium-2-aldoxime methanesulphonate (P2S). In a previous paper in the series it was reported that small amounts of thiocyanate are formed in rats treated with P2S.[2] As it is known that many nitriles are converted to thiocyanate in the body experiments were now performed to see if this also happens with MPN.

MATERIALS AND METHODS

N-methylpyridinium-2-nitrile methanesulphonate (*MPN*) was synthesized as follows: 5·2 g pyridine-2-nitrile and 5·7 g methyl methanesulphonate were solved in benzene and heated under reflux for 2 hr. The solid mass obtained after cooling was washed with dry ether and the product recrystallized from absolute ethanol. A weight of 5·2 g (yield: 50 per cent) of a white, very hygroscopic substance was obtained. The melting point (determined on a Kofler heating stage) was 128 °C. (Found: C, 44·8; H, 4·61. Calc. for $C_8H_{10}N_2O_3S$: C, 44·8; H, 4·67).

MPN was measured by a procedure based on the determination of cyanide by Zinke-Königs reaction following the rapid hydrolysis of the nitrile to cyanide in alkaline solution. Thiocyanate, which interferes in the colorimetric step, was removed by silver precipitation before hydrolysis of the sample. The analysis was carried out as follows: Urine was treated with $AgNO_3$ and NaCl as described in the previous paper.[2] To 0·4 ml of the supernatant was added 3·2 ml water and 0·4 ml 1 M NaOH. Ten minutes later 0·8 ml 1 M NaH_2PO_4 and 0·2 ml 1% Chloramine T solution were added, followed after 1 min by 1 ml of barbituric acid pyridine reagent (3 g barbituric acid + 1·6 ml HCl (s.g. 1·19) and 16 ml pyridine diluted with water to 50 ml final volume*). The absorbancy at 580 mμ was determined from 7 to 15 min later. The

* This is the reagent used in Ref. 3, but the composition of the reagent is incorrectly described in this reference.

MPN content of the sample was obtained from a standard graph. It was verified that the hydrolysis of MPN is complete within 1 min under these conditions and that MPN added to normal urine was quantitatively recovered by the analytical procedure. Thiocyanate and the cyanide-forming metabolite were determined as described previously.

The identity of MPN in the urine from P2S treated animals was established by paper chromatography. Ascending development on Whatman no. 1 was used. The spots were revealed by a colour reaction based on the hydrolysis of MPN to cyanide and the immediate reaction of the latter with a modified copper–phenolphthalin reagent[4]* The colour, red spots on white background, was developed by spraying the paper with a mixture of equal volumes of 0·1 M KOH and a solution prepared by mixing 2·5 ml 1% phenolphthalin in ethanol and 50 ml 0·1% $CuSO_4 \cdot 5H_2O$ in water and diluting this mixture to 100 ml with water.

The LD_{50} determinations were performed on inbred mice of both sexes weighing 20 ±1 g. The calculations were made according to Miller and Tainter.[5] Solutions were freshly prepared in distilled water and the injected volume was 10 ml/kg body weight.

RESULTS

Rats were given P2S and the urinary excretion of MPN was then determined. The results (Table 1) shows that about 8 per cent of the orally administered and 4 per cent

TABLE 1. MPN DETERMINATIONS ON URINE FROM NORMAL AND P2S TREATED RATS

Two rats received 120 μmoles P2S i.m. and two 400 μmoles orally The 24-hr urine was collected and assayed for MPN.

Dose of P2S	μmoles MPN/24 hr	
	Control	After P2S
400 μmoles *per os*	<0·2	31·5
	<0·2	30·8
120 μmoles i.m.	<0·2	4·8
	<0·2	4·6

of the intramuscularly injected P2S was excreted as MPN during the first 24-hr interval. Paper chromatography of the urine from P2S treated rats demonstrated the presence of a nitrile with the following R_f-values: acetone–water (3:1) R_f 0·65; *n*-butanol–acetic acid–water (4:1:1) R_f 0·43 and phenol–water (4:1) R_f 0·91. The R_f values were found to be identical with those of an authentic specimen of MPN.

P2S was also administered orally to human volunteers and the urinary excretion of MPN determined. As shown in Fig. 1, a significant excretion of MPN was detected but the amount found during the first 2 days corresponded only to about 0·3 per cent of the P2S administered. Thus, considerably less MPN was excreted in human beings than in rats.

Of interest in this connection is the toxicity of MPN and P2S. The LD_{50} ±s.e. (after i.p. injection in mice) was found to be 75 ±3 mg/kg and 132 ± 4 mg/kg, respectively.

* Thiocyanate did not respond to this test. It was not possible to detect cyanide on paper chromatograms.

As many nitriles are converted to thiocyanate *in vivo*, rats were injected with MPN and the urine analysed for thiocyanate by the two methods used previously.[2] The results are shown in Fig. 2.

From these results it is evident that thiocyanate and a compound which behaves as metabolite X formed in P2S treated animals[2] are found in the urine from MPN

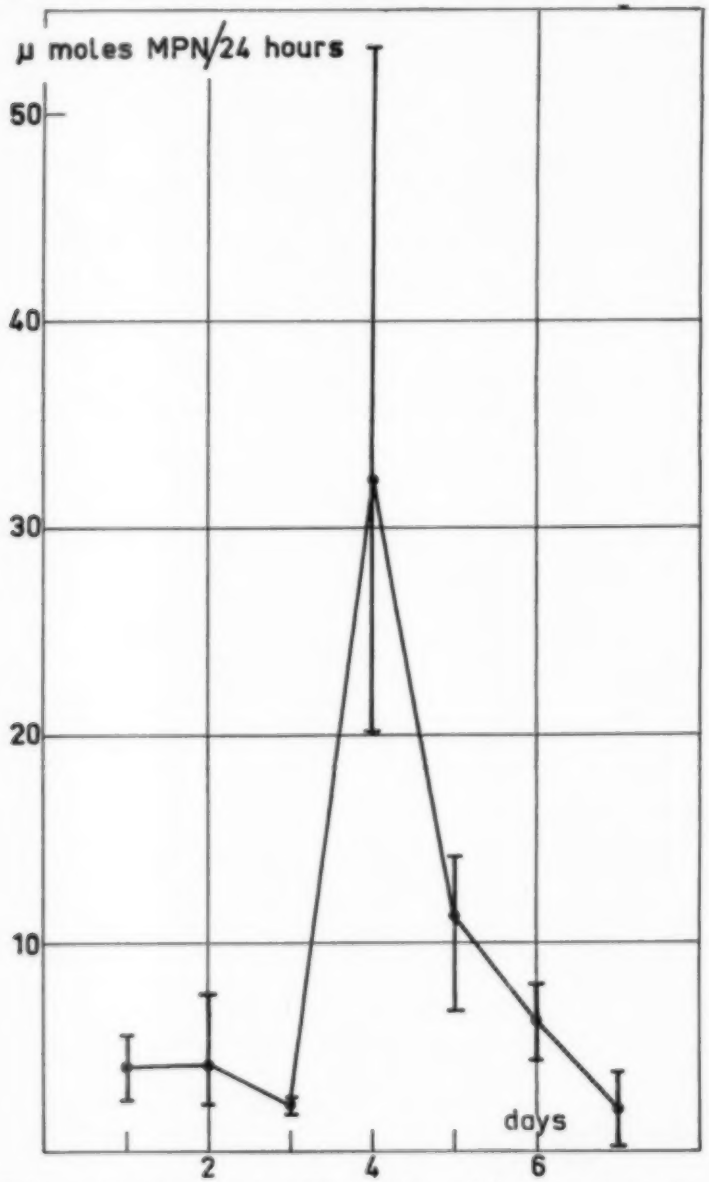


FIG. 1. MPN determinations on urine from P2S treated human beings. Four human volunteers were given 3 g of P2S orally on day 3.

Vol. 7
1961

treated animals.* The amounts found corresponded to from 6 to 7 per cent each of thiocyanate and metabolite X.

With a tenfold smaller dose of nitrile the amounts of thiocyanate and metabolite X found in the first 24-hr period were 5 and 10 per cent, respectively.

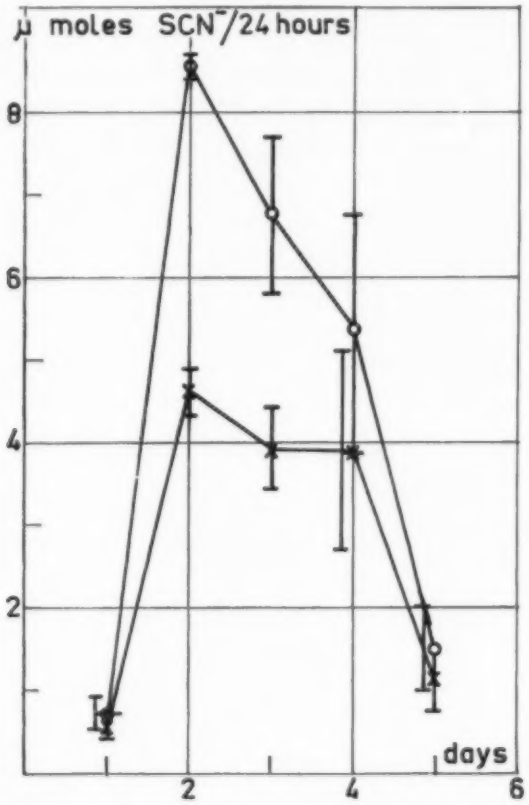


FIG. 2. Thiocyanate determinations on urine from MPN treated rats. Two rats received an intramuscular injection of 120 μmoles of MPN (corresponding to 90 mg/kg) on day 1. ○——○ Method I (thiocyanate plus unknown metabolite). +——+ Method II true thiocyanate.

DISCUSSION

The results presented in this paper show that thiocyanate and possibly metabolite X are formed from MPN. Since it has also been demonstrated that MPN is formed from P2S *in vivo* it is very likely that the nitrile is the precursor of both thiocyanate and metabolite X. Agreeing with this hypothesis is the fact that man excretes less nitrile and forms less thiocyanate and metabolite X after administration of P2S than do rats.

Although MPN is slightly more toxic than P2S it cannot significantly contribute to the toxicity of P2S as only a small fraction of P2S is converted to MPN.

* Traces of cyanide (less than 0·5 μmoles/24 hr) were also found in these urine samples.

The conversion of MPN to thiocyanate is of interest, as no heterocyclic nitriles have been studied from this point of view. It may be mentioned that aliphatic, but not aromatic, nitriles are converted to thiocyanate in the body.[6]

Acknowledgement—Grateful appreciation is expressed to Mr. G. Engström and I. Hedenborg for their able technical assistance.

REFERENCES

1. R. ELLIN, *J. Amer. Chem. Soc.* **80**, 6588 (1958).
2. I. ENANDER, A. SUNDWALL and B. SÖRBO, *Biochem. Pharmacol.* 7, 226 (1961).
3. C. NYSTRÖM and B. SÖRBO, *Scand. J. Clin. Lab. Invest.* **9**, 223 (1957).
4. W. A. ROBBIE, *Arch. Biochem.* **5**, 49 (1944).
5. L. C. MILLER and M. L. TAINTER, *Proc. Soc. Exp. Biol., N.Y.* **57**, 261 (1944).
6. R. T. WILLIAMS, *Detoxification Mechanisms* (2nd Ed.), p. 404. Chapman & Hall, London (1959).

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 237–247. Pergamon Press Ltd., Printed in Great Britain.

THE PHARMACOLOGY OF KCN AS A PROPHYLACTIC AGAINST RADIATION*

C. VAN DER MEER and P. W. VALKENBURG

Medical Biological Laboratory of the National Defence Research Organization TNO, Lange Kleiweg 139, Rijswijk (Z.H.), The Netherlands

(*Received* 24 *March* 1961)

Abstract—A dose of 100 μg of KCN protects about 80 per cent of mice against a lethal dose of total body irradiation; on lowering the dose the protection decreases rapidly. A protective dose of KCN markedly reduces the oxygen tension in the spleen and bone marrow of these mice. The same dose reduces the total oxygen consumption of the animal to about 25 per cent. The respiration is slowed considerably but the respiratory volume shows little change. The oxygen saturation of the arterial blood is unaltered, the saturation of the venous blood is increased. After an initial fall with about 50 mm Hg the blood pressure rises again but remains about 20 mm below normal.

Concurrently with the hypoxia in the spleen and bone marrow the oxygen tension in the brain is augmented considerably. It is concluded that KCN protects against radiation by causing hypoxia in the blood-forming organs. This hypoxia is probably caused by a severe local vasoconstriction due to intense vasomotor stimulation.

INTRODUCTION

IN A previous paper[1] it was shown that a number of biologically active amines which protected mice against a dose of X-rays which otherwise would kill the animals by inactivation of the blood-forming organs, decreased the oxygen tension in the spleen of these mice at protective doses. As a relation was found between the percentage survival of the mice and the percentage reduction of the oxygen tension in the spleen it was concluded that the protection afforded by these compounds was caused by their ability to lower the oxygen tension in the spleen, and possibly also in other blood-forming organs, thereby rendering these organs less sensitive to radiation. This does not imply that all protective compounds act by this same mechanism. In order to explain the prophylactic effect of cyanide a number of hypotheses were put forward.

Bacq *et al.*[2] who discovered the prophylactic effect of cyanide in mice, at first assumed that it acted either by decreasing the metabolic activity or by inhibition of peroxydase. Later Alexander *et al.*[3] suggested that the effect of cyanide might be explained by inactivation of HO_2 radicals.

Cohen *et al.*[4] postulated that the inhibition of cytochrome oxidase by cyanide might be responsible for its protective activity. They assumed that due to this inhibition some link in the metabolic chain, representing the radiosensitive site, was kept in the reduced form and was therefore less sensitive to radiation.

Recently van den Brenk and Moore[5] suggested that cyanide might protect by respiratory inhibition followed by anoxic anoxaemia.

* A preliminary report has been published in *Acta Physiol. Pharmacol. Neerl.* **9**, 324 (1960).

If inhibition of cytochrome oxidase in the tissues were the important feature one might expect that cyanide can protect mice while the oxygen tension in the blood-forming organs is normal or even increased. This would have interesting theoretical implications since, because of the highly specific action of cyanide, the radiosensitive site would then be open to direct biochemical investigation.

However, our experiments showed that KCN injected into mice at protective doses lowered the oxygen tension in the spleen to a sufficient extent to explain its action by a mechanism identical with that of the biologically active amines. Further experiments to be described in this paper were directed towards the elucidation of the mechanisms by which this reduction in oxygen tension was caused in our mice at doses that protected these animals against irradiation.

METHODS

Experimental animals

All animals used were females of our highly inbred CBA/Rij strain weighing 18–20 g.

Irradiation and estimation of protective activity*

This was performed in exactly the same way as described in a previous paper.[1] All animals received 675 r, a dose which causes over 98 per cent mortality in the control animals. Protective activity was expressed as the percentage 30 days' survival.

Estimation of the oxygen tension in the spleen, brain and bone marrow

Oxygen tension in the spleen was estimated as described in a previous paper.[1] In order to estimate the oxygen tension in the brain a 100-μ platinum wire was pushed through an opening in the skull into the right hemisphere over a distance of 1–2 mm. The localization of the electrode was not standardized. The electrode was connected to the surface of the skull by dentist's cement (Justi Resin Cement) and exteriorized on the back of the neck. The oxygen tension in the bone marrow was estimated by pushing a 50μ platinum electrode into the right femur after a small hole was drilled in the bone between the upper and middle third of the shaft. The electrode was pushed into the marrow cavity over a distance of 2 mm. The electrode was fastened by a thread around the femur just above the hole. It was exteriorized at the back of the mouse.

Determination of the oxygen consumption of the intact animal

The oxygen consumption was determined in a closed system. The changes in volume were measured every minute at atmospheric pressure using a 50-ml burette filled with water. A special device was necessary to inject the animal with KCN while in the closed system. This apparatus will be described in detail elsewhere. In principle it consists of part of a plastic syringe connected by means of Sterivac no. 1 polythene tubing to the peritoneal cavity. The plunger of this syringe is pulled in by two rubber bands. The rubber bands are stretched by means of a small piece of a match placed between a knob on the plunger and a Perlon thread between two small pillars on the

* We are grateful to Dr. D. W. van Bekkum for performing the irradiation experiments. Recent measurements by Dr. Joh Blok have shown that the radiation doses presented in this and previous papers must be multiplied by 1·14 in order to obtain the midline dose in roentgens for mice. This corrected dose is considered to differ less than 4 per cent from the tissue dose. We prefer to maintain our original dose value in this paper for the convenience of comparing our present results with those presented in previous papers.

end of the syringe. Around one of these pillars a 100-μ platinum wire is wound which is connected to a copper antenna. When a high-frequency signal is applied from the outside the platinum wire is heated, the Perlon thread melts and the plunger is released. This device is fastened to the back of the animal and the canula brought into the peritoneum under light ether anaesthesia at least 1 hr before the experiment. Apparently the animal is not in the least disturbed by the presence of this apparatus on its back as it does not try to remove it and behaves quite normally.

Determination of the respiratory volume

Under urethane anaesthesia (0·5 g/kg intraperitoneally, 0·5 g/kg subcutaneously) a canula consisting of a piece of nylon tube N_1 (Talas, Zwolle, Holland) was put into the trachea. This canula was connected to a glass vessel containing 290 ml of air. The pressure variations in this container were recorded with a pressure transducer (capacitive displacement meter, Van Reysen, Delft, Holland) and an Ediswan pen writer. The air in the container was changed every 5 min. The curves were magnified photograpically about five times and the surface under the curve measured with a planimeter over a distance corresponding to 4 sec. As only changes in respiratory volume were of interest the respiratory volume is expressed as cm²/min. The respiration frequency could be read directly from the curve.

Determination of the oxygen saturation of the blood

Either the left carotic artery or the right jugular vein were canulated in animals under urethane anaesthesia (0·5 g/kg intraperitoneally, 0·5 g/kg subcutaneously). The arterial canula consisted of a piece of Sterivac no. 1 drawn out at the tip over a small piece of no. 20 hypodermic needle. The venous canula was just a piece of Sterivac no. 1.

Before the experiment 0·1 ml of heparin Vitrum (diluted to 67 i.u./ml) was injected through the canula. Heparin Vitrum (5000 i.u./ml) was diluted twenty times with a saponin solution (Merck) containing 0·2 g/ml in distilled water. This solution was brought to the 0·05-ml mark into a 1-ml tuberculin syringe containing two small glass spheres. After a few drops of blood were drawn from the canula the syringe was filled with blood until the solution reached the 0·2-ml mark. The contents were mixed well by means of the glass spheres. Then 0·1 ml was removed and the remainder poured into a Perspex cuvette with a light path of about 0·2 mm. The density at 560 mμ and at the isobestic point 505 mμ was read in a Beckman model B spectrophotometer and the ratio D560/D505 calculated. As the results from the experiments were perfectly clear in themselves no determinations of fully oxygenated and fully reduced blood were made which would enable the exact calculation of the percentage oxygen saturation corresponding with each ratio of D560/D505. For comparison it may be mentioned that for rat blood a difference of 0·1 in this ratio corresponds to a difference of about 10 per cent in the oxygen saturation.

Determination of the blood pressure

For the determination of the blood pressure in the unanaesthetized mouse the same method was used as described previously.[1] An "average blood pressure" was recorded by using a slow recording galvanometer.

KCN

Fresh solutions of KCN in distilled water were made up immediately before each experiment. These solutions were not neutralized as even careful neutralization with HCl caused considerable variation in the cyanide content.

RESULTS

The protection of KCN *against radiation*

Three doses of KCN were injected intraperitoneally into groups of mice just before irradiation with a lethal dose of X-rays (675 r). The effect of this treatment on the 30-days' survival is presented in Table 1.

TABLE 1. THE EFFECT OF KCN INJECTED INTRAPERITONEALLY JUST PRIOR TO IRRADIATION OF FEMALE CBA/RIJ MICE OF ABOUT 20 G WITH A DOSE OF 675 R OF X-RAYS

Dose (μg)	Number of mice	30-days' survival	Percentage protected
100	70	57/70	81
75	40	12/40	30
50	40	3/40	8
—	40	0/40	0

Table 1 shows that 100 μg of KCN, which is very close to a lethal dose, gives good protection. At lower doses the protection decreases rapidly.

The effect of KCN *on the oxygen tension in the spleen and bone marrow*

The same doses of KCN were used to study the effect of KCN on the oxygen tension in the spleen of unanaesthetized mice. The current flowing at 0·6 V between a 100 μ platinum electrode in the spleen (negative side) and a silver–silver chloride electrode in the rectum was measured before and after the administration of KCN. Percentage reduction of oxygen tension was calculated as

$$100 - \frac{\text{current after KCN}}{\text{current before KCN}} \times 100$$

The time between the intraperitoneal injection and the moment at which the current had returned to its preinjection value was also noted. The results are shown in Table 2.

TABLE 2. THE EFFECT OF KCN ON THE OXYGEN TENSION IN THE SPLEEN OF UNANAESTHETIZED FEMALE CBA/RIJ MICE OF ABOUT 20 G

Dose (μg)	Number of mice	Average maximal reduction of oxygen tension (% $\pm$ s.d.)	Duration of the effect (min $\pm$ s.d.)
100	17	82 $\pm$ 3·3	30 $\pm$ 2·9
100*	9	83 $\pm$ 4·7	24 $\pm$ 2·6
75	11	84 $\pm$ 4·3	21 $\pm$ 2·0
50	13	72 $\pm$ 5·0	15 $\pm$ 1·9

* KCN injected 20 min after 1 g/kg urethane (0·5 g intraperiteoneally, 0·5 g subcutaneously).

Doses of 100 and 75 μg per animal reduce the oxygen tension by about 80 per cent, and the effect of 50 μg is possibly somewhat smaller. Although the magnitude of the effect is not much different for the three doses used, the duration of the effect decreases considerably with the dose. As the time interval between the injection and the end of the irradiation lies between 17 and 20 min in our irradiation experiments, the duration of the effect may become a significant feature determining the amount of protection. The group receiving 100 μg of KCN under urethane anaesthesia was added because a number of experiments to be described below could not be performed on unanaesthetized animals and were made under urethane anaesthesia. In eight unanaesthetized mice receiving 100 μg of KCN the oxygen tension in the bone marrow was estimated. The average maximal reduction of the oxygen tension was 78 $\pm$ 4·4 per cent and the average duration of the effect 24 $\pm$ 1·9 min, in good correspondence with the effect in the spleen.

Although we do not claim that our measurements are quantitative our impression is that the oxygen tension in the bone marrow is not significantly lower than in the spleen. The relatively low values found by Cater and Silver[6] in normal human bone marrow may be explained by the difficulties of the technique, differences in the site of placements of the electrode or differences in the animal species investigated.

The effect of KCN *on the oxygen consumption of the intact animal*

The three doses of KCN were used for studying their effect on the oxygen consumption of the intact animal. The oxygen uptake was measured every minute. The average uptake in ml per min at 0 °C and 760 mm Hg over periods of 10 min was calculated for each animal. Means and standard deviations of the 10-min averages for the animals in each dose group are presented in Table 3.

Table 3 shows that a marked reduction of the oxygen consumption down to about 25 per cent of the control value is caused by 100 μg of KCN. Even 80 min after the injection the oxygen consumption is still below normal. For the lower doses of KCN the inhibition of the oxygen consumption is somewhat smaller but especially the duration of the effect is much shorter.

The effect of KCN *on respiratory volume and frequency of respiration*

In order to perform our pharmacological experiments under conditions as closely similar as possible to those under which the animals were irradiated it was attempted to derive the respiratory volume from measurements of intrapleural pressure in the unanaesthetized mouse. This method does not offer too many technical difficulties, but a comparison of the changes in intrapleural pressure with pressure changes in a closed system connected to the trachea under urethane anaesthesia, showed that the relation between the values obtained with both methods which was constant in normal animals, differed considerably after the injection of KCN. Therefore our experiments had to be performed on animals in urethane anaesthesia in which the trachea was canulated. As it was shown in Table 2 that urethane anaesthesia has little effect on the changes in oxygen tension in the spleen the results of experiments under urethane anaesthesia appear to give adequate information. The effect of 100 μg of KCN on respiratory volume and respiration frequency are presented in Tables 4 and 5. These tables show a very marked decrease in respiration frequency to less than a third of the original value accompanied by somewhat erratic changes in respiratory volume. Only one of the four experiments shows a considerable reduction of the respiratory volume.

TABLE 3. THE AVERAGE OXYGEN CONSUMPTION (ML/MIN) OF FEMALE CBA/RIJ MICE OF ABOUT 20 G BEFORE AND AFTER INTRAPERITONEAL INJECTION OF KCN

(Averages of 10-min periods.)

KCN (μg)	Number of mice	Average oxygen consumption over 10-min periods ± s.d.									
		Before KCN		After KCN							
		20–10	10–0	0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80
100	5	1·72 ± 0·04	1·72 ± 0·07	0·41 ± 0·04	0·38 ± 0·03	0·45 ± 0·06	0·60 ± 0·11	0·71 ± 0·19	0·77 ± 0·20	0·92 ± 0·18	1·11 ± 0·07
75	4	1·56 ± 0·03	1·50 ± 0·05	0·51 ± 0·01	0·64 ± 0·04	0·85 ± 0·11	1·22 ± 0·17	1·39 ± 0·05	1·39 ± 0·02	—	—
50	6	1·52 ± 0·09	1·47 ± 0·10	0·58 ± 0·03	1·02 ± 0·11	1·29 ± 0·17	1·39 ± 0·11	1·35 ± 0·12	1·43 ± 0·10	—	—

Vol. 7
1961

It should be noted that the variation in the values for the normal respiratory volumes is caused by differences in the setting of the recording apparatus.

The effect of KCN *on the oxygen saturation of arterial and venous blood*

The oxygen saturation was determined as the ratio of the optical density at 560 mμ over the density at 505 mμ (D560/D505) in haemolysed blood. Only two

TABLE 4. INSPIRATORY VOLUME (IN CM^2/MIN, SEE METHODS) BEFORE AND AFTER INTRAPERITONEAL INJECTION OF 100 μG KCN

Female CBA/RIJ mice about 20 g. urethane 1 g/kg (0·5 g intraperitoneally, 0·5 g subcutaneously)

Mouse no.	Respiratory volume (cm^2/min)			
	Before	2 min after	5 min after	10 min after
1	150	225	128	—
2	405	210	195	225
3	360	360	375	630
4	285	375	210	525

TABLE 5. RESPIRATION FREQUENCY OF THE MICE OF TABLE 4

Mouse no.	Respirations per min			
	Before	2 min after	5 min after	10 min after
1	180	75	45	—
2	210	105	75	150
3	285	60	60	75
4	210	180	60	150

determinations were made on each animal either in arterial or in venous blood. One determination was made before KCN, the other at different intervals after intraperitoneal injection of 100 μg of KCN as shown in Table 6.

It is apparent from Table 6 that the saturation of the arterial blood is practically unaltered whereas the saturation of the venous blood is increased as shown by a decreased ratio D560/D505. Therefore there is no reason to assume that the decrease in oxygen tension in the spleen is caused by insufficient oxygenation of the blood.

The effect of KCN *on the blood pressure*

The blood pressure was recorded in unanaesthetized mice. Four doses of KCN were used. The blood pressure after KCN was compared with the value before KCN and the difference in mm Hg averaged over the group of mice. The results are presented in Table 7.

A dose of 25 μg has very little effect except for a small rise in blood pressure. The higher doses produce an initial fall of about 50 mm Hg after which the pressure very soon either returns to normal (50 μg) or remains for some time about 20 mm below the pre-injection level. During these experiments the heart rate was also recorded. However, the results are so erratic that it is impossible to draw any conclusion from them.

It is felt that the relatively small fall in blood pressure apparent from the experiments of Table 7 is not sufficient in itself to cause the considerable reduction of oxygen tension noted in the spleen. Presumably this can only be caused by vasoconstriction either as a compensation for the fall in blood pressure or as a result of stimulation of the vasomotor centre by KCN itself.

TABLE 6. THE EFFECT OF INTRAPERITONEAL INJECTION OF 100 μG KCN ON THE OXYGEN SATURATION, EXPRESSED AS D560/D505 OF ARTERIAL AND VENOUS BLOOD OF FEMALE CBA/RIJ MICE ABOUT 20 G

(Urethane 1 g/kg (0·5 g intraperitoneally, 0·5 g subcutaneously.))

Before KCN (*A*)	Arterial blood			
	After 100 μg KCN (*B*)			Difference (*B*–*A*)
	3 min	5 min	10 min	
1·82	1·82			0
1·78	1·78			0
1·80	1·80			0
1·82		1·82		0
1·81		1·80		−0·01
1·81		1·85		+0·04
1·79		1·82		+0·03
1·82			1·83	+0·01
1·83			1·83	0
	Venous blood			
2·35	2·21			−0·14
2·18	2·00			−0·18
2·08	2·05			−0·03
2·19		2·00		−0·19
2·22		2·01		−0·21
2·11		2·01		−0·10
2·24			2·00	−0·24
2·20			2·01	−0·19

TABLE 7. THE EFFECT OF KCN ON THE BLOOD PRESSURE OF UNANAESTHETIZED FEMALE CBA/RIJ MICE OF ABOUT 20 G

(Average deviations from the pre-injection values are given in mm Hg.)

KCN (μg)	Number of mice	Average change in blood pressure after KCN (mm Hg)					
		1 min	2 min	5 min	10 min	20 min	40 min
100	10	−53	−25	−23	−18	−12	−4
75	7	−46	−23	−20	−17	− 6	−5
50	6	−48	− 7	− 3	+ 5	0	–
25	8	− 4	+ 8	+14	+ 4	− 2	–

We were unable to determine the blood flow through the mouse spleen, but it was assumed that if KCN acted on the vasomotor centre the oxygen tension in the brain would not be reduced since the brain vessels are considered to be non-reactive to a vasomotor stimulus.

The effect of KCN *on the oxygen tension in the brain*

We felt that the implantation of an electrode in the brain simultaneously with an electrode in the spleen was too heavy a strain for a mouse. Therefore the oxygen tension in the brain was recorded in a number of separate experiments. The results of sixteen experiments with 100 μg KCN are presented in Table 8.

Although the results show a large degree of variation the general trend is clearly that a very short initial fall is followed by a substantial rise of much longer duration.

The initial decrease is probably caused by the large initial fall in blood pressure whereas the secondary rise corresponds in its duration to the reduction of the oxygen tension found in the spleen.

TABLE 8. THE EFFECT OF 100 μG KCN ON THE OXYGEN TENSION IN THE BRAIN OF UNANAESTHETIZED FEMALE CBA/RIJ MICE OF ABOUT 20 G

Exp. no.	Initial fall (%)	Duration (min)	Secondary rise (%)	Duration (min)
1	69	4	53	18
2	88	2	11	20
3	32	4	150	30
4	19	2	67	52
5	33	6	0	—
6	17	2	56	20
7	32	6	8	21
8	20	2	350	27
9	21	2	125	30
10	17	2	83	34
11	27	2	73	35
12	0	—	92	34
13	86	3	93	35
14	10	3	60	20
15	0	—	138	26
16	10	2	200	40
average $\pm$ s.d.	29 $\pm$ 7	2·6 $\pm$ 0·43	97 $\pm$ 22	28 $\pm$ 5

In a few rats of about 200 g the oxygen tension in the spleen and brain were measured simultaneously before and after 500 μg of KCN. In these experiments the similarity in time course of the two effects was clearly demonstrated.

DISCUSSION

The experiments described in this paper show that doses of KCN which protect mice against a lethal dose of total body irradiation reduce the oxygen tension in the spleen and bone marrow of unanaesthetized mice under conditions comparable to the irradiation experiments. In a previous paper[1] experiments were described in which the reduction of the oxygen tension in the spleen caused by injection of a number of protective amines as well as by a lowering of the oxygen content of the inspired air, was compared with the protection against radiation afforded by these experimental procedures. These experiments strongly indicate that for the compounds studied the protection against radiation is mediated by a reduction of the oxygen tension in a critical blood-forming organ.

When the results obtained with KCN are compared with these previous experiments it appears that the values for the 100 μg dose fit into the curve showing the relation

between percentage protection and percentage reduction of oxygen tension in the spleen. However, at lower doses of KCN the protection is somewhat less than might be expected from the reduction of the oxygen tension in the spleen. A possible explanation of this discrepancy may be offered by the fact that the duration of the effect was not taken into account in the construction of the curve showing the relation between protection and reduction of oxygen tension. Especially after 50 μg of KCN the effect on the spleen is over before the end of the period of irradiation. On the other hand a radiomimetic effect of cyanide has been described in a number of biological objects. For instance Read[7] described an inhibition of growth, while Lilly and Thoday[8] described an inhibition of mitosis and an increase in the number of chromosome aberrations in *Vicia faba* roots. Kihlman[9] also investigating *Vicia faba* roots described the formation of chromosome breakages by KCN and Kihlman *et al.*[10] showed that the number of chromosome breakages caused by X-rays was increased by KCN under anaerobic conditions. Wagner *et al.*[11] showed that cyanide increases the mutation frequency in Neurospora crassa, whereas Sobels[12] found that cyanide increases the number of sex-linked lethal mutations caused by X-rays in *Drosophila*. A similar effect in the mouse superimposed on the protection caused by hypoxia might tend to decrease the protective capacity.

Whatever may be the explanation of this slight discrepancy there is no reason to assume that the protection afforded by KCN to mice is due to a mechanism other than reduction of the oxygen tension in the spleen and bone marrow. Evidently these experiments give no indication as to the way in which this hypoxia causes protection at the cellular level.

The reduction of the oxygen tension in the spleen is somewhat unexpected as it is known that due to the inhibition of cytochrome oxidase the oxygen uptake in the tissues is lowered and as a consequence the oxygen saturation of the venous blood is increased. The experiments reported in the present paper show accordingly that at the protective doses used, the total oxygen consumption of the animal is greatly reduced and the saturation of the venous blood is increased while the arterial blood shows a normal saturation.

As it was shown that the release of oxygen from the haemoglobin is not affected by KCN,[13] the hypoxia in the spleen could only be caused by a substantial impairment of the blood supply to this organ. According to Pilcher and Sollmann[14] an intense stimulation of the vasomotor centre is seen after KCN. Although we were unable to demonstrate vasoconstriction in the different organs directly, we could show that the oxygen tension in the brain increased considerably concurrent with the decrease in the spleen. This is in accordance with the view that KCN stimulates the vasomotor centre in a similar way as it is stimulated during asphyxia, causing vasoconstriction in all organs which are sensitive to a vasomotor stimulus (such as the spleen) but leaving a number of organs (such as the brain) unaffected.

In the spleen the hypoxia caused by vasoconstriction then overcompensates the reduced oxygen uptake due to cytochrome-oxidase inhibition whereas in the brain the cytochrome-oxidase inhibition, possibly combined with an increased blood flow, becomes apparent as a rise in oxygen tension.

The only experimental fact that does not fit into this picture of vasomotor stimulation, is the fall in blood pressure where a rise was to be expected. Possibly this fall

in blood pressure is caused by a vasodilatation in the mesenteric vessels as was observed by Bean and Sidky[15] on local application of cyanide. This vasodilatation then might reduce the venous return. On the other hand other possibilities such as vasodilitation in different parts of the body (e.g. the brain) or a direct action on the heart muscle cannot be excluded by the present experiments. Konstantinova and Grayevsky[16] found a reduction of the oxygen tension in the spleen not exceeding 25 per cent after 150 μg of KCN was injected subcutaneously. Possibly this smaller effect may be explained either by differences in the strains of mice used or by the fact that these authors injected the compound subcutaneously while we used the intraperitoneal route. In accordance with the slight reduction of the oxygen tension in the spleen they found no protection against a dose of 900 r of ^{60}Co γ-rays.

REFERENCES

1. C. van der Meer and D. W. van Bekkum, *Int. J. Rad. Biol.* **1**, 5 (1959).
2. Z. M. Bacq, A. Herve, J. Lecomte and P. Fisher, *Science* **111**, 356 (1955).
3. P. Alexander, Z. M. Bacq, S. F. Cousens, M. Fox, A. Herve and J. Lazar, *Rad. Res.* **2**, 392 (1955).
4. J. A. Cohen, O. Vos and D. W. van Bekkum, *Proceedings of the Fifth International Conference on Radiobiology, Stockholm* (1956).
5. H. A. S. van den Brenk and R. Moore, *Radiation Biology, Proceedings of the Second Australasian Conference on Radiation Biology, London* (1959).
6. D. B. Cater and I. A. Silver, *Acta Radiol.* **53**, 233 (1960).
7. J. Read, *Radiation Biology of Vicia Faba, Oxford* (1959).
8. L. J. Lilly and J. M. Thoday, *Nature, Lond.* **177**, 338 (1956).
9. B. A. Kihlman, *J. Biophys. Biochem. Cytol.* **3**, 363 (1957).
10. B. A. Kihlman, T. Merz and C. P. Swanson, *J. Biophys. Biochem. Cytol.* **3**, 381 (1957).
11. R. P. Wagner, C. H. Haddox, R. Fuerst and W. S. Stone, *Genetics* **35**, 237 (1950).
12. F. H. Sobels, *Nature, Lond.* **177**, 979 (1956).
13. C. L. Evans, *J. Physiol.* **53**, 17 (1919).
14. J. D. Pilcher and T. Sollmann, *J. Pharmacol.* **6**, 361 (1914–1915).
15. J. W. Bean and M. M. Sidky, *Amer. J. Physiol.* **189**, 541 (1957).
16. M. M. Konstantinova and E. J. Grayevsky, *Dokl. Akad. Nauk SSSR* **132**, 1427 (1960).

Biochemical Pharmacology, 1961, Vol. 7, pp. 248–255. Pergamon Press Ltd., Printed in Great Britain.

METABOLIC STUDIES ON THE RELEASE OF HISTAMINE BY COMPOUND 48/80 IN THE RAT DIAPHRAGM*

A. M. ROTHSCHILD, I. VUGMAN AND M. ROCHA E SILVA

Department of Pharmacology, Faculty of Medicine of Ribeirão Prêto, Univ. S. Paulo, Ribeirão Prêto, Est. S. Paulo, Brasil

(*Received* 6 *March* 1961)

Abstract—The inhibitory action of uncoupling agents like 2:4-dinitrophenol, salicylate, thiopental and sodium oleate on the release of histamine from isolated rat diaphragm by compound 48/80, is markedly decreased by glucose. Inhibitions due to sodium cyanide or anoxia are similarly affected. Rat mesentery mast cell damage by 48/80 *in vitro*, which is suppressed by dinitrophenol or sodium cyanide, will occur if the action of these inhibitors is tested in the presence of glucose. Sodium succinate cannot prevent the inhibition of histamine release by dinitrophenol, although oxygen consumption studies reveal that it is aerobically metabolized by dinitrophenol-treated tissue. The results obtained have been interpreted as an indication that metabolic intermediates necessary for the release of histamine from rat diaphragm can be generated by a mechanism, possibly glycolytic, functioning independently of the Krebs cycle.

Vol. 7
1961

INTRODUCTION

A CORRELATION between active metabolism and the release of histamine was first indicated by Parrot,[1] who demonstrated that the liberation of histamine induced by the antigen in sensitized guinea-pig tissue did not take place in the absence of oxygen. Efforts to clarify the mechanism of histamine release, by looking for a possible link between cell metabolism and the response of the tissue to antigen, have been made by several investigators. Working with minced guinea-pig lung preparations, Mongar and Schild[2] have shown that SH-group reagents, sodium cyanide, low temperature, low pH, and calcium lack, as well as pre-incubation at 45 °C, effectively prevent *in vitro*-release of histamine. Partial inhibitions were also obtained with antipyretics like salicylate, acetanilide, etc. Although some of these compounds can function as inhibitors of oxidative phosphorylation, Mongar and Schild noted that a potent uncoupling agent like 2:4-dinitrophenol (DNP), was ineffective in blocking histamine release by antigen, even when tested at levels (50 mM) which are 300 times higher than those required to uncouple oxidative phosphorylation in liver mitochondrial preparations.[3] These results were at variance with those reported by Moussatché and Danon[4]; using guinea-pig lung slices from sensitized animals, they reported that 0·1 mM DNP, as well as 0·01 mM pentachlorophenol, effectively blocked the release of histamine by antigen. The same authors[5] demonstrated that histamine release from guinea-pig lung could be appreciably increased by the addition of Krebs cycle intermediates to the Ringer–Barron fluid employed as incubation medium. They postulated that the

* A preliminary report of these results has been presented at the XII Annual Meeting of the Brazilian Society for the Advancement of Science, on 9–12 July, 1960, in Piracicaba, E.S. Paulo, Brazil, and published in *Ciência e Cultura* **12**, 99 (1960).

release of histamine in guinea-pig anaphylaxis is dependent on energy-rich phosphorylated compounds supplied by aerobic oxidative metabolism.[4]

Following the discovery that several amines of relatively simple structure have histamine-releasing properties, use has been made of such compounds for the study of the histamine-releasing process. In 1949, Rocha e Silva and Schild,[6] using the isolated rat diaphragm, studied the histamine-releasing action of *d*-tubocurarine. Examining the influence of temperature on this process they found that, besides its dependence on physical factors like the diffusion of *d*-tubocurarine and histamine through the tissue, the action of the alkaloid seemed to have a chemically controlled phase. Using minced guinea-pig lung, Mongar and Schild[2] have studied the release of histamine by compound 48/80 and other amines, but their results have shown that in this tissue amines are unsuitable models for the study of histamine release in anaphylaxis.

The high potency of compound 48/80 in inducing the release of histamine from rat tissue[7, 8] as well as the sensitivity of the process to temperature[9] and metabolic inhibitors,[10] has been stressed by Rocha e Silva.[11] Using the isolated rat diaphragm technique, Rocha e Silva *et al.*[9] have shown that the histamine-releasing action of compound 48/80 is affected by inhibitors like salicylate, calcium lack, low pH, etc., in much the same manner as is the release of histamine in guinea-pig lung anaphylaxis. Similar results have been obtained more recently by Mota *et al.*,[12] who have shown that the release of histamine from rat tissue by *in vitro*-anaphylaxis or by compound 48/80 was sensitive to the same agents used by Mongar and Schild[2] to inhibit this release in guinea-pig lung anaphylaxis.

The conflicting results regarding the inhibitory effects of dinitrophenol on histamine release,[2, 4] could have been caused by differences in the incubation conditions employed. It was therefore considered of interest to reinvestigate this problem using the isolated rat diaphragm technique and 48/80 as the histamine-releasing agent.

MATERIALS AND METHODS

Compound 48/80, a condensation product of *p*-methoxiphenethyl methylamine and formaldehyde, was obtained from the Wellcome Research Laboratories, Tuckahoe, N.Y. Krebs–Ringer phosphate buffer, pH 7·3, was prepared using one-half of the originally recommended concentration of $CaCl_2$ suggested by Umbreit.[13]

Wistar rats of either sex, weighing from 150 to 200 g, were stunned and decapitated and their diaphragms excised. After removing fat and loose connective tissue, the diaphragms were cut into from 5 to 8 pieces of about 80 mg wet weight each. One piece was used in each incubation flask.

Incubations were performed at 37 °C in air, in 10 × 100 mm test tubes shaken by a back-and-forth movement of about 300 strokes/min. All samples were pre-incubated for 30 min at 37 °C in buffer or in the presence of inhibitor, or substrate, or both. After adding the releasing agent, incubations were continued for 25 min. Final vols. were 2·0 ml. Controls and blanks containing, respectively, either no inhibitor or no releasing agent, were always run in parallel with the experimental incubations.

In the experiments with sodium oleate, pre-incubations were performed in Krebs–Ringer buffer prepared without calcium and magnesium. Prior to the addition of the 48/80 the tissue samples were transferred to the complete Krebs–Ringer medium but containing no oleate. This procedure apparently did not interfere with the release of

histamine in the controls; it was employed to avoid interaction of oleate with calcium and magnesium ions during pre-incubation.

Following incubation, tissues were removed, gently blotted, weighed and placed in 0·1 N HCl. Residual histamine was extracted in the manner described by Feldberg and Talesnik.[8] Incubation media containing released histamine were kept frozen until the moment of assay.

Histamine was assayed on the atropinized ileum of the guinea-pig; the tissue was suspended in Tyrode's solution at 37 °C. Comparisons were made with standard solutions of histamine phosphate. Some of the inhibitors employed affected the response of the ileum to this standard. Prior to assay such compounds were removed by acidification and ether extraction (thiopental), or acidification and boiling (NaCN).

Oxygen consumption was measured in the Warburg apparatus. Approximately 100 mg of rat diaphragm were placed in the main compartment of the flasks, and buffer containing the substrate was added. The centre-well contained 20% KOH. After a 10-min equilibration period, the solution of DNP was added from the side-arm. Consumption of oxygen was measured during the 30- to 60-min interval which followed the addition of DNP. Of compound 48/80, 20 μg per ml, added from the second side-arm, were present during this period.

Experiments in anoxia were performed in Thunberg tubes. The nitrogen employed was freed from oxygen by passing it over incandescent copper filings, previously reduced by exposure to a stream of hydrogen. Oxygen in the incubation medium and in the tissue was removed by alternately evacuating and filling the tube with nitrogen.

TABLE 1. RELEASE OF HISTAMINE FROM ISOLATED RAT DIAPHRAGM BY COMPOUND 48/80
Influence of glucose and other metabolites on the inhibition induced by 2:4-dinitrophenol (DNP).

Addition to medium	Per cent histamine released	
	Total	Total – blank
—	43·6 ± 2·7* (6)	38·3
Glucose, 4·5 mM	51·3 ± 2·8 (6)	45·2
DNP, 0·03 mM	35·5 ± 3·8 (6)	31·0
DNP, 0·03 mM + glucose, 4·5 mM	43·5 ± 3·2 (6)	39·0
DNP, 0·3 mM	8·5 ± 2·9 (4)	4·8
DNP, 0·3 mM + glucose, 4·5 mM	48·2 ± 5·6 (4)	43·7
DNP, 0·3 mM + succinate, 5 mM	8·3 ± 1·1 (3)	3·8
DNP, 0·3 mM + fumarate, 5 mM	4·1 ± 2·2 (3)	2·1
DNP, 0·3 mM + acetate, 5 mM	4·6 ± 1·6 (2)	2·3
DNP, 0·3 mM + pyruvate, 5 mM	3·5 ± 2·8 (3)	1·5
DNP, 0·3 mM + D-xylose, 4·5 mM	8·7 ± 1·8 (3)	3·4

Figures within parentheses refer to the number of experiments performed.
* Standard error of the mean 48/80, 20 μg/ml.

RESULTS

Results are expressed as percentage of the total histamine of tissue released during incubation. The first column of figures of Table 1 shows that an average of 43·6 per cent of the histamine of the tissue sample is released when the tissue is incubated, in glucose-free buffer, with 20 μg of 48/80 per ml. A small but significant increase to 51·2 per cent ($P < 0·05$) was observed when 4·5 mM glucose was present during incubation. The second column of figures represents the net release of histamine. It was obtained by subtracting values for the spontaneous release of histamine (blanks)

from the data in the first column. In the experiments presented in this work, blank values ranged between 4 and 11 per cent of the total histamine present in the tissue.

Table 1 shows that 0·03 mM DNP added to glucose-free buffer induced a moderate but significant reduction ($P = 0{\cdot}05$) in the amounts of histamine released by 48/80. When assayed in the presence of 4·5 mM glucose, inhibition by DNP was no longer noticeable. The effect of glucose was shown to a much greater extent in experiments performed in the presence of 0·3 mM DNP. As indicated in Table 1, at this level DNP produced almost complete suppression of the histamine-releasing action of compound 48/80. In this case again, 4·5 mM glucose practically abolished the effect of DNP. Table 1 shows also that Krebs cycle metabolites like succinate, fumarate and pyruvate, as well as D-xylose, did not reverse the action of 0·3 mM DNP.

The relation between glucose concentration and the suppression of DNP-induced inhibition of the release of histamine is shown in Fig. 1. It can be seen that abolition

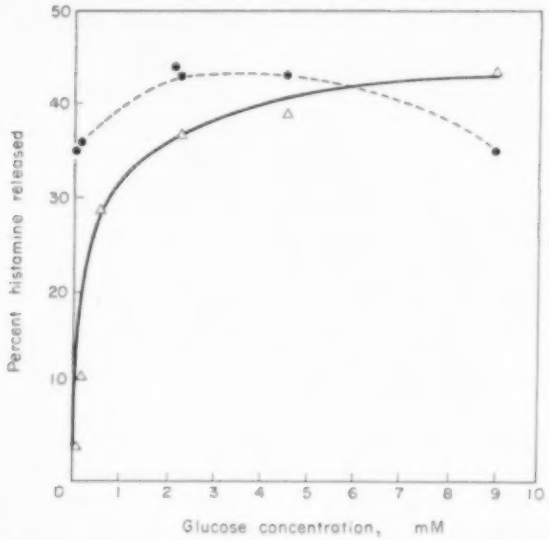


FIG. 1. Influence of glucose concentration on the DNP-induced inhibition of the release of histamine from rat diaphragm by compound 48/80. △——△: incubations performed in the presence of 0·3 mM DNP. ●——●: controls, incubated in the absence of DNP. Each point is the average of three experiments and is corrected for blank values.

of the action of DNP was obtained at glucose levels above 5 mM, but that lower concentrations of glucose were already effective. The second curve in this figure indicates that, even in the absence of DNP, glucose exerted a slight potentiation of the action of compound 48/80. This effect was no longer apparent at 10 mM glucose.

The uncoupling action of DNP in most tissues is usually associated with an increased oxygen consumption, which may be followed by a phase of decreased oxygen uptake. At higher concentrations of DNP, the latter effect often predominates and a depressed respiratory activity may be noted soon after the tissue came into contact with the inhibitor.[14] In order to investigate the effects of DNP on oxygen consumption of rat diaphragm, experiments were made in the Warburg apparatus. Table 2 shows that in an atmosphere of air, 0·03 mM DNP increased oxygen uptake by rat diaphragm both

in the presence and absence of glucose. At the 0·3 mM level, DNP induced a significant depression of oxygen consumption, the degree of which was slightly reduced by the presence in the medium of 4·5 mM glucose; this indicated that the tenfold increase in histamine release (shown in Table 1), was accompanied by a much smaller increase in overall oxygen consumption. The effect of succinate on oxygen consumption in the presence of 0·3 mM DNP was also studied. Table 2 shows that it increased oxygen uptake to levels obtained in the absence of DNP. When compared with the results obtained with succinate (Table 1), it becomes apparent that in this case there was again no correlation between oxygen consumption and histamine release by 48/80. The effects on oxygen consumption of 0·3 mM DNP, of glucose and of succinate were also examined in an atmosphere of pure oxygen. Table 2 shows that this environment did not change the relative values of oxygen uptake, although it increased these values in all samples.

TABLE 2. OXYGEN CONSUMPTION OF DNP-TREATED RAT DIAPHRAGM

Addition to medium	Oxygen consumption*	
	In air	In oxygen
—	11·4 ± 1·4† (5)	
Glucose, 4·5 mM	11·0 ± 0·8 (4)	17·7 ± 3·4 (3)
Succinate, 5 mM	11·3 ± 1·3 (5)	17·3 ± 3·3 (3)
DNP, 0·03 mM	16·2 ± 1·2 (3)	
DNP, 0·03 mM + glucose	18·0 ± 1·3 (3)	
DNP, 0·3 mM	4·2 ± 0·8 (5)	6·4 ± 0·6 (3)
DNP, 0·3 mM + glucose	6·2 ± 0·4 (4)	7·8 ± 0·7 (3)
DNP, 0·3 mM + succinate	12·8 ± 2·3 (5)	21·9 ± 6·1 (2)

Figures within parentheses indicate the number of experiments performed.
* μl/min per g wet weight.
† Standard error of the mean.

In a complex system such as the rat diaphragm, in which many different types of cells contribute to overall oxygen consumption, results like those presented on Table 2 give only incomplete evidence about the correlation between aerobic metabolism and the release of histamine by compound 48/80. It was felt that a clearer picture of the pathway by which glucose affected the histamine-releasing process would be obtained by examining its effects on tissue under anoxia or treated with sodium cyanide. These conditions can be expected to inhibit aerobic metabolism extensively in most mammalian cells. The results shown in Table 3 indicate that 1 mM cyanide inhibited the release of histamine by 48/80, as already shown by Mota.[12] Nitrogen anoxia was also inhibitory, although to a lesser extent. The presence of 4·5 mM glucose effectively reversed both inhibitions; however, a residual inhibition of about 20 per cent remained in both cases. In experiments in which from 2·25 mM to 9·0 mM glucose was used, this residual inhibition was not abolished.

Previous work in this laboratory [9] had indicated that salicylate inhibited the release of histamine from rat diaphragm incubated with compound 48/80 in buffered physiological saline. Salicylate also inhibits histamine release in antigen–antibody reactions,[2] and by anaphylatoxin;[15] its action as an uncoupling agent has been described.[16] Table 4 shows the effects of salicylate, in the presence and absence of glucose, on the release of histamine by 48/80; 25 mM salicylate was strongly inhibitory; in the presence

Vol. 7
1961

of glucose its action was depressed, a 30 per cent inhibition remaining. At the 10 mM level salicylate was also inhibitory; however, its action was almost completely reversed by the presence of glucose.

Sodium oleate and thiopental, although having no structural similarity to uncouplers like DNP or salicylate, have been described as having similar effects on mitochondria.[17, 3] The action of these two agents on the release of histamine by 48/80 is also shown in Table 4. The inhibition produced by thiopental was of the order of 70 per cent; it was markedly decreased by the presence of glucose. Sodium oleate, 1mM, inhibited the release of histamine to the same degree as thiopental; as with the barbiturate, the action of oleate was extensively reversed by glucose.

TABLE 3. RELEASE OF HISTAMINE FROM ISOLATED RAT DIAPHRAGM BY COMPOUND 48/80
Influence of glucose on the inhibition induced by anoxia and by sodium cyanide.

Addition to medium	Per cent histamine released	
	Total	Total – blank
—	45·2 ± 3·1* (6)	41·3
NaCN, 1 mM	5·9 ± 1·2 (6)	2·0
NaCN, 1 mM + glucose, 4·5 mM	36·7 ± 1·7 (6)	32·8
None (aerobic controls)	42·0 ± 2·8 (5)	36·7
Nitrogen atmosphere	25·1 ± 3·8 (5)	14·1
Nitrogen atmosphere + glucose, 4·5 mM	40·6 ± 2·1 (5)	29·6

* Standard error of the mean 48/80, 20 μg/ml.
Figures within parentheses indicate the number of experiments performed.

TABLE 4. RELEASE OF HISTAMINE FROM ISOLATED RAT DIAPHRAGM BY COMPOUND 48/80
Influence of glucose on the inhibition produced by sodium salicylate, sodium oleate and thiopental.

Addition to medium	Per cent histamine released	
	Total	Total – blank
—	50·0 ± 4·3* (5)	43·8
Salicylate, 25 mM	8·3 ± 1·8 (3)	2·0
Salicylate, 25 mM + glucose, 4·5 mM	36·5 ± 2·2 (4)	30·2
Salicylate, 10 mM	20·4 ± 1·6 (3)	14·5
Salicylate, 10 mM + glucose, 4·5 mM	43·0 ± 7·0 (2)	36·8
—	46·7 ± 4·5 (3)	38·2
Thiopental, 1 mM	21·8 ± 4·7 (3)	13·5
Thiopental, 1 mM + glucose, 4·5 mM	43·7 ± 2·9 (3)	35·2
—	52·0 ± 6·5 (4)	44·5
Oleate, 1 mM	20·7 ± 4·2 (4)	13·2
Oleate, 1 mM + glucose, 4.5 mM	55·6 ± 7·8 (4)	48·1

* Standard error of the mean 48/80, 20 μg/ml.
Figures within parentheses indicate the number of experiments performed.

Mast cells alterations

Typical morphological alterations of rat tissue mast cells are evoked by 48/80.[18] The effects of metabolic inhibitors on this action have been studied by Junqueira and Beiguelman[19] who, among other compounds, found DNP and NaCN to prevent the

extrusion of granules which is induced by 48/80. We have confirmed these results, but have found the inhibitory action of DNP to be evident to a marked extent only in the absence of glucose. Using pieces of rat mesentery, microscopic examination of the results of incubations with 20 μg of 48/80 per ml in Krebs–Ringer phosphate buffer revealed that: (a) in samples pre-incubated with 0·3 mM DNP, 6 per cent of the mast cells showed granule extrusion; (b) this value was increased to 70 per cent when 4·5 mM glucose plus 0·3 mM DNP were present; and (c) controls incubated in the absence of either compound presented granule extrusion in 99 per cent of the cells. Similar experiments using 1 mM sodium cyanide showed: (a) in controls, 95 per cent of the cells presented granule extrusion; (b) in the presence of NaCN this value was reduced to 8 per cent; and (c) NaCN plus 4·5 mM glucose revealed effects of 48/80 in 87 per cent of the cells. Each result refers to averages of two experiments, each performed in triplicate with samples of mesentery from one animal. A total of approximately 300 cells were counted in each sample.

DISCUSSION

The results presented in this paper indicate that the release of histamine from isolated rat diaphragm by compound 48/80 is effectively blocked by 2:4-dinitrophenol. The presence of glucose, but not of succinate, pyruvate, acetate or fumarate, reversed the inhibitory effect of DNP. The increased oxygen consumption of DNP-treated tissue, observed in the presence of succinate, indicated that this substrate was being metabolized, and yet was unable to affect the inhibition of the histamine-releasing system. Accordingly, it appeared that glucose was affecting the DNP-inhibition by a route other than the Krebs cycle and the cytochrome electron transport chain. The possiblity that glucose reversed the action of DNP by a direct chemical interaction was considered unlikely. D-Xylose, a compound having a similar chemical activity as glucose, but which is less readily metabolized by the mammalian cell,[20] was ineffective in relieving histamine release from inhibition by DNP.

A phenomenon similar to that encountered with DNP, was observed when glucose was used in the presence of two other inhibitors of the release of histamine by 48/80, namely, sodium cyanide and nitrogen anoxia. These inhibitors, although having another site of action than DNP, will ultimately affect aerobic cell metabolism in a similar way, e.g. by depressing the synthesis of high-energy phosphate. The ability of glucose to reverse the inhibition of histamine release by these agents, as well as by DNP and uncoupling agents like salicylate, thiopental or oleate, could be explained most logically by the suggestion that the sugar enables the cell to use other metabolic pathways to provide the histamine-releasing process with high-energy intermediates. Randle[21] has pointed out that, like insulin, inhibitors of oxidative phosphorylation can stimulate glucose uptake by rat muscle *in vitro* and probably have similar effects *in vivo*. A relationship between the release of histamine and the effects of insulin *in vivo* has been indicated by the results of Goth *et al.*;[22] these showed that in alloxan-diabetic rats the liberation of histamine by dextran or egg-white is severely depressed, but takes place normally following insulin treatment. It seems possible that in tissue treated with inhibitors of oxidative phosphorylation, the glycolytic breakdown of glucose supplies the metabolites required by the histamine-releasing process. A high glycolytic activity under aerobiosis has been noted in leucocytes.[23] In view of the similarity between tissues mast cells and the leucocytic basophils[24, 25] the search for

Vol. 1
1961

the relative importance of glycolysis in mast cell metabolism and especially in the amine-releasing mechanism are interesting possibilities for future work.

Results of studies[26] on the effects of DNP and glucose on histamine release in guinea-pig lung anaphylaxis have shown that the powerful inhibition of this process evoked by 0·3 mM DNP, although significantly sensitive to partial reversal by glucose, was less affected than DNP-inhibition of the release of histamine by compound 48/80 in rat diaphragm. In contrast, experiments on rat anaphylaxis have revealed that in the isolated sensitized diaphragm, the release of histamine by antigen, which is also abolished by 0·3 mM DNP, is not only reversed, but markedly potentiated when glucose plus DNP are present in the medium.

Thus, it becomes apparent that the study of the action of metabolic substrates like glucose or others on the effects of inhibitors of histamine release, may well provide a valuable tool for the clarification of the biochemical events which accompany the release of amines in animal tissues.

Note added in proof

Upon completion of this manuscript it was learned that B. Westerholm (*Acta Physiol. Scand.* **50**, 300, 1960) had shown that the *in vitro* release of histamine produced by 48/80 in cat skin is blocked by anoxia, and that glucose reverses this block. The author also refers to unpublished results by Diamant, in which effects of glucose and anoxia, similar to those described by us on Table 4, were obtained.

REFERENCES

1. J. L. Parrot, *C.R. Soc. Biol., Paris* **136**, 361 (1942).
2. J. L. Mongar and H. O. Schild, *J. Physiol.* **135**, 301, 320 (1957).
3. F. E. Hunter, *Methods in Enzymology* (Edited by S. P. Colowick and N. O. Kaplan) Vol. II, p. 601. Academic Press, New York, 1955.
4. H. Moussatché and A. P. Danon, *Experienta*, **14**, 414 (1958).
5. H. Moussatché and A. P. Danon, *Arch. Biochem. Biophys.* **77**, 108 (1958).
6. M. Rocha e Silva and H. O. Schild, *J. Physiol.* **109**, 448 (1949).
7. W. D. M. Paton, *Brit. J. Pharmacol.* **6**, 499 (1951).
8. W. Feldberg and J. Talesnik, *J. Physiol.* **120**, 550 (1953).
9. M. Rocha e Silva, I. Vugman and A. Antonio. Unpublished results.
10. N. Chakravarty, *Acta Physiol. Scand.* **48**, 146 (1960).
11. M. Rocha e Silva, *Ann. Acad. Bras. Ciênc.* **31**, 77 (1959).
12. I. Mota and T. Ishii, *Brit. J. Pharmacol.* **15**, 82 (1960).
13. W. W. Umbreit, R. H. Burris and J. F. Stauffer, *Manometric Techniques and Tissue Metabolism.* Burgess Publ. Co. (1957).
14. D. B. Tyler, *J. Biol. Chem.* **184**, 711 (1950).
15. C. G. Haining, *Brit. J. Pharmacol.* **11**, 107 (1956).
16. S. W. Jeffrey and M. J. H. Smith, *Biochem. J.* **72**, 462 (1959).
17. B. C. Pressman and H. A. Lardy, *Biochim. et Biophys. Acta* **21**, 458 (1956).
18. I. Mota, W. T. Beraldo and L. C. U. Junqueira, *Proc. Soc. Exp. Biol., N.Y.* **83**, 455 (1953).
19. L. C. U. Junqueira and B. Beiguelman, *Tex. Rep. Biol. Med.* **13**, 69 (1955).
20. D. M. Kipnis and C. F. Cori, *J. Biol. Chem.* **224**, 681 (1957).
21. P. J. Randle, *Acta Endocrinol.* **34**, Suppl. 50, 175 (1960).
22. A. Goth, W. L. Nash, M. Nagler and J. Holman, *Amer. J. Physiol.* **191**, 25 (1957).
23. S. P. Martin, G. R. McKinney and R. Green, *Ann. N.Y. Acad. Sci.* **59**, 996 (1955).
24. A. W. A. Boseila, *Proc. Soc. Exp. Biol., N.Y.* **97**, 184 (1958).
25. H. T. Graham, O. H. Lowry, F. Wheelwright, M. A. Lenz and H. H. Parish, Jr., *Blood* **10**, 467 (1955).
26. A. M. Rothschild and M. Rocha e Silva. To be published.

Biochemical Pharmacology, 1961, Vol. 7, pp. 256–264. Pergamon Press Ltd., Printed in Great Britain.

ACTION OF THIAMINE AND ITS ANALOG ON NEUROMUSCULAR TRANSMISSION IN THE CAT*

S. H. NGAI, S. GINSBURG AND R. L. KATZ†

Departments of Anesthesiology, Neurology and Pharmacology,
College of Physicians and Surgeons, Columbia University, New York, New York

(*Received* 23 *January* 1961)

Abstract—The action of thiamine and a number of its analogs on tibialis twitch response to peroneal nerve stimulation, respiration, and arterial pressure, as well as their effect on the neuromuscular block produced by *d*-tubocurarine and decamethonium, were studied in anesthetized cats.

It was found that thiamine, pyrithiamine, and pyrithiamine analogs in which there is a hydroxy group on the pyridinium ring caused a decrease in the twitch without initial potentiation, depressed respiration, and lowered arterial pressure. These compounds also antagonized both *d*-tubocurarine and decamethonium.

On the other hand, methyl thiazolium iodide, methyl pyridinium iodide, and the analogs of thiamine and pyrithiamine in which there is no hydroxy group on the ring bearing the quarternary nitrogen, produced an increase in the twitch with muscular fasciculation, and with larger doses, a decrease in the twitch. Changes in respiratory movements generally paralleled those of the twitch. Arterial pressure was usually elevated. These compounds antagonized *d*-tubocurarine and potentiated decamethonium.

The thiazolium fragment, at the dose level studied, increased the twitch only slightly. It also antagonized *d*-tubocurarine and potentiated decamethonium. The pyrimidine fragment seemed to have no effect on the twitch.

It appears that the activity of thiamine and pyrithiamine on the neuromuscular junction is related to the quarternary nitrogen, the "pyrimidyl" coupling, and the presence of a hydroxy group on the onium-bearing ring. The antagonistic action of these compounds against *d*-tubocurarine and decamethonium also depends on the same structural components.

THE action of thiamine in blocking neuromuscular transmission has been reported by several groups of investigators.[1–4] Cheymol *et al.*[5] and di Palma and Hitchcock[6] have extended study of this action of thiamine to include some of its derivatives.

In the cat, thiamine antagonized decamethonium, succinylcholine and *d*-tubocurarine, but, with successive doses, finally potentiated *d*-tubocurarine.[5] On the other hand, in the rat diaphragm preparation *in vitro*, thiamine consistently potentiated *d*-tubocurarine, decamethonium and succinylcholine.[4]

The study here reported undertook to re-examine the action of thiamine on the neuromuscular transmission and its influence on the block produced by *d*-tubocurarine

* Supported by the National Institute of Neurological Diseases and Blindness, USPHS, Grant no. B31C; the Division of Research Grants and Fellowships, National Institutes of Health, Grant no. B573; and Burroughs Wellcome and Co. (U.S.A.), Inc.

† Postdoctoral trainee, Division of General Medical Sciences, National Institutes of Health under Grant no. 2G-56 to the Department of Anesthesiology.

and decamethonium, using the cat peroneal nerve–tibialis preparation. In addition, pyrithiamine, oxythiamine, and a number of their analogs were similarly tested. This study further attempted to correlate the structures of these compounds with their pharmacologic activities.

METHODS

Eighty-nine cats, each weighing between 2 and 4 kg, were used. The animals were anesthetized with 36 mg/kg of pentobarbital sodium administered intraperitoneally. The peroneal nerve was separated from the sciatic trunk, ligated at the mid-thigh level, and a shielded Palmer bipolar electrode applied to its peripheral end. At 10-sec intervals, electric stimuli consisting of rectangular pulses of 0·1-msec duration and 3–5-V intensity were delivered to the nerve by a Grass stimulator (model S4C) via a stimulus isolation unit. The resulting twitch responses of the tibialis muscle were measured with a Grass force displacement transducer (model FT-03, loaded with 300-g springs). Arterial pressure was measured with a Statham transducer (P23A) from the femoral artery on the other side. An accordion type pneumograph placed around the lower thorax registered respiratory movements. All recordings were made on a Grass model 5 polygraph. When necessary, artificial ventilation was provided with a Palmer ideal pump.

All drugs were injected intravenously through an in-dwelling catheter in a femoral vein. When the action of thiamine or its analogs was investigated in the presence of *d*-tubocurarine or decamethonium, one of the latter two was injected first to produce a partial or a complete paralysis of short duration, and one of the former was then injected during the recovery phase. When several compounds were tested in a single animal, an interval of at least 1 hr was provided between the paired injections. The sequence of testing these compounds was varied from animal to animal.

Table 1 depicts the structures of compounds tested in the study. Compounds other than thiamine, pyrithiamine, and oxythiamine were synthesized in the laboratory of the Department of Neurology by one of the authors (S.G.).*

* *Methods of synthesis*

Method 1. Methyl pyridinium iodide was prepared in the usual manner by reacting pyridine with methyl iodide under cooling.

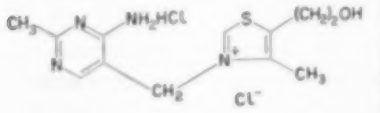
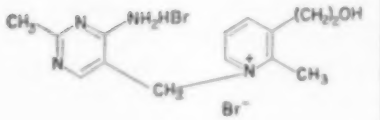
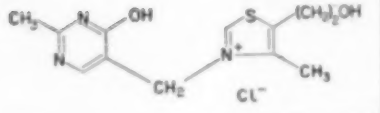
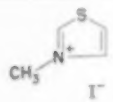
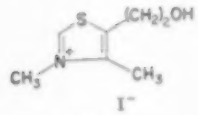
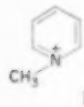
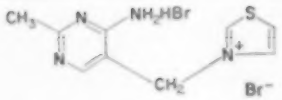
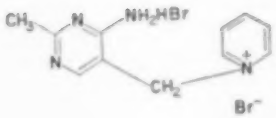
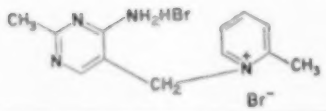
Methyl thiazolium iodide and 3:4-dimethyl-5-β-hydroxyethyl thiazolium iodide were prepared by refluxing for 30 min a solution of the corresponding base in dimethylformamide with an excess methyl iodide. Precipitation of the quaternary salt was completed through addition of absolute ether. For preparation of 4-methyl-5-β-hydroxyethyl thiazole from commercial thiamine see Williams *et al.*[7]

Method 2. 4-Amino-2-methyl-5-bromomethyl pyridine hydrobromide, which was used for the preparation of the quaternary compounds, was kindly supplied by Dr. Karl Pfister of Merck & Co., Inc. Five millimoles of the "pyrimidine" were dissolved in 12 ml of dimethylformamide and the corresponding base was added using a 40–50 per cent excess. After a short while, precipitation of the quaternary salt started; the mixture was left at room temperature for 1 day, the solids were filtered off and dissolved in boiling methanol. When the solution had cooled a small amount of absolute ether was added to promote precipitation of the pure salt. Yields ranged from 40 to 60 per cent of the theoretical.

For the preparation of 1-(4-amino-2-methyl pyrimidyl-5-methyl)-3-hydroxy pyridinium bromide hydrobromide, 3-acetoxy pyridine (prepared by acetylation of 3-pyridol with acetic anhydride) was used for quaternization. The crude compound was subsequently de-acetylated by refluxing for 1 hr with an aqueous-methanolic solution of HBr (about 1·5 M), evaporated to dryness, and finally recrystallized from methanol–ether. Melting of all compounds except the thiazole derivative were too high to be determined.

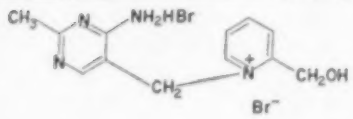
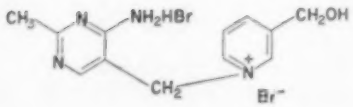
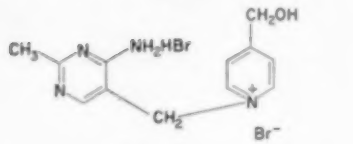
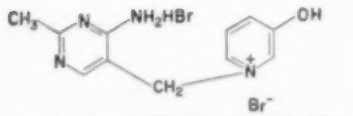
Analysis. Halogen content was determined by the Volhard method and neutral equivalents were measured by potentiometric titration.

TABLE 1.

Structural formulae	Names	M.P. (°C)	Method of synthesis*
	Thiamine HCl, 3-(4-amino-2-methyl pyrimidyl-5-methyl)-4-methyl-5-β-hydroxyethyl thiazolium chloride hydrochloride (M.W. = 337)	248 (decomp)	commercial
	Pyrithiamine HBr, 1-(4-amino-2-methyl pyrimidyl-5-methyl)-2-methyl-3-β-hydroxyethyl pyridinium bromide hydrobromide (M.W. = 420)	218–220	commercial
	Oxythiamine Cl, 3-(4-hydroxy-2-methyl pyrimidyl-5-methyl)-4-methyl-5-β-hydroxyethyl thiazolium chloride (M.W. = 302)	195 (decomp.)	commercial
	Methyl thiazolium iodide (M.W. = 227)	151–152	1
	3:4-Dimethyl-5-β-hydroxyethyl thiazolium iodide (M.W. = 285)	—	1
	Methyl pyridinium iodide (M.W. = 221)	116	1
	3-(4-Amino-2-methyl pyrimidyl-5-methyl)-thiazolium bromide hydrobromide (M.W. = 368)	247	2
	1-(4-Amino-2-methyl pyrimidyl-5-methyl)-pyridinium bromide hydrobromide (M.W. = 362)	—	2
	1-(4-Amino-2-methyl pyrimidyl-5-methyl)-2-methyl pyridinium bromide hydrobromide (M.W. = 376)	—	2

Vol. 7
1961

TABLE 1.—*continued.*

Structural formulae	Names	M.P. (°C)	Method of synthesis*
	1-(4-Amino-2-methyl pyrimidyl-5-methyl)-2-hydroxymethyl pyridinium bromide hydrobromide (M.W. = 392)	—	2
	1-(4-Amino-2-methyl pyrimidyl-5-methyl)-3-hydroxymethyl pyridinium bromide hydrobromide (M.W. = 392)	—	2
	1-(4-Amino-2-methyl pyrimidyl-5-methyl)-4-hydroxymethyl pyridinium bromide hydrobromide (M.W. = 392)	—	2
	1-(4-Amino-2-methyl pyrimidyl-5-methyl)-3-hydroxy pyridinium bromide hydrobromide (M.W. = 378)	—	2

* See footnote under Methods.

RESULTS

To facilitate the presention of experimental findings and discussion, the compounds are divided into four categories:

(A) Thiamine, pyrithiamine and oxythiamine.
(B) Fragments of thiamine and pyrithiamine.
(C) Thiamine and pyrithiamine analogs in which there is no hydroxy group on the ring bearing the quarternary nitrogen.
(D) Pyrithiamine analogs in which there is a hydroxy group on the pyridinium ring.

A. *Thiamine, pyrithiamine and oxythiamine*

1. *Effect on tibialis twitch response, respiration and arterial pressure.* Thiamine hydrochloride in doses up to 10 mg/kg had no effect on the tibialis twitch response to stimulation of the peroneal nerve. In two of four instances, 20 mg of thiamine per kg caused a transient, minimal decrease in the twitch. Administration of 40 mg or more of thimaine per kg resulted in complete or nearly complete paraylisis and apnea, both effects lasting for 15–20 min. The decrease in the twitch was never preceded by an increase, nor was muscular fasciculation ever observed (Fig. 1). In two preparations, 0·02 mg of neostigmine methylsulfate per kg partially antagonized the action of thiamine.

The arterial pressure fell by 40–50 mm Hg with 5 mg of thiamine per kg. With larger doses, hypotension was more pronounced. Recovery was gradual, requiring 10–15 min to reach the pre-injection level or a steady level lower than that of the control.

Four milligrams of pyrithiamine hydrobromide per kg resulted in a slight and transient decrease in the twitch response; 40 mg/kg reduced the twitch to approximately 50 per cent of control. The respiratory movements were depressed, requiring the institution of artificial respiration. With the dose levels studied, there was a moderate drop of arterial pressure.

Oxythiamine hydrochloride, in doses up to 50 mg/kg, had no effect on twitch response and respiration. However, it produced a fall in arterial pressure comparable to that caused by thiamine.

2. *Effect on neuromuscular block produced by* d-*tubocurarine and decamethonium.* In ten instances, thiamine was administered during the recovery phase of neuromuscular block produced by *d*-tubocurarine. In seven of these a dose of thiamine of from 2 to 10 mg/kg resulted in a definite increase in the twitch response (Fig. 2, top) and in the remaining three, 10 mg of thiamine per kg potentiated the action of *d*-tubocurarine.

Pyrithiamine and oxythiamine, in doses of 5–15 mg/kg, also antagonized *d*-tubocurarine but were less potent than thiamine.

Thiamine, pyrithiamine, and oxythiamine, in doses of 5–15 mg/kg, regularly antagonized the action of decamethonium (Fig. 2, bottom). In no instance was potentiation observed even after repeated administration of these compounds.

B. *Fragments of thiamine and pyrithiamine.*

Methyl thiazolium iodide, 3:4-dimethyl-5-β-hydroxyethyl thiazolium iodide, methyl pyridinium iodide, and 4-amino-2-methyl-5-bromomethyl pyrimidine hydrobromide.

1. *Effect on tibialis twitch response, respiration, and arterial pressure.* Both methyl thiazolium iodide and methyl pyridinium iodide increased the twitch response at dose levels of 1–10 mg/kg and 4–20 mg/kg, respectively. With larger doses (20 mg/kg and 40 mg/kg, respectively) an initial increase in the twitch and muscular fasciculation was followed by partial paralysis. Usually both the rate and amplitude of respiration increased, except that after 40 mg of methyl pyridinium iodide per kg, the respiration became depressed after initial stimulation. The arterial pressure was markedly elevated (Fig. 3).

3:4-Dimethyl-5-β-hydroxyethyl thiazolium iodide in doses of 10–50 mg/kg increased the twitch response slightly. The repiration was not affected significantly. The arterial pressure showed a fall of from 20 to 50 mm Hg.

4-Amino-2-methyl-5-bromomethyl pyrimidine hydrobromide in doses up to 50 mg/kg had no apparent effect on twitch response and respiration. There was a slight fall in arterial pressure.

2. *Effect on neuromuscular block produced by* d-*tubocurarine and decamethonium.* Methyl thiazolium iodide, methyl pyridinium iodide and 3:4-dimethyl-5-β-hydroxyethyl thiazolium iodide, in decreasing order of potency, antagonized *d*-tubocurarine and potentiated decamethonium (Fig. 4).

4-Amino-2-methyl-5-bromomethyl pyrimidine hydrobromide had no influence on the block produced by either *d*-tubocurarine or decamethonium.

Vol. 7
1961

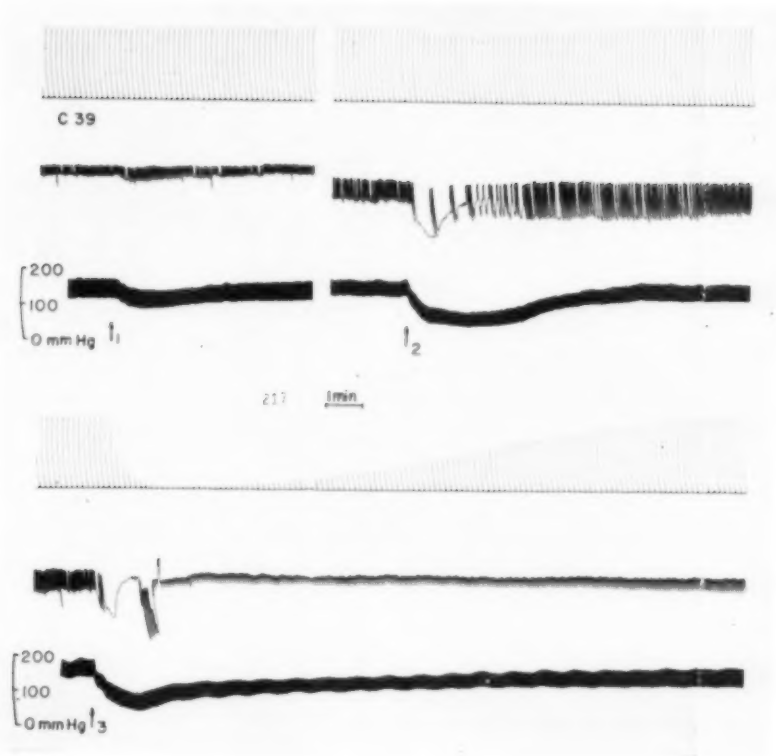


FIG. 1. Cat 39, 3 kg Pentobarbital anesthesia, 36 mg/kg, given intraperitoneally. *Top trace*, tibialis muscle twitch response to peroneal nerve stimulation; *middle trace*, respiratory movements; *bottom trace*, arterial pressure. Same anesthesia and parameters for all subsequent figures except those of the middle and bottom traces, which may be in reverse order. At arrow 1, 10 mg/kg; at arrow 2, 20 mg/kg and at arrow 3, 60 mg/kg of thiamine, intravenously. Respiration failed within 1 min after the last injection. Artificial respiration continued to the end of tracings.

facing page 260

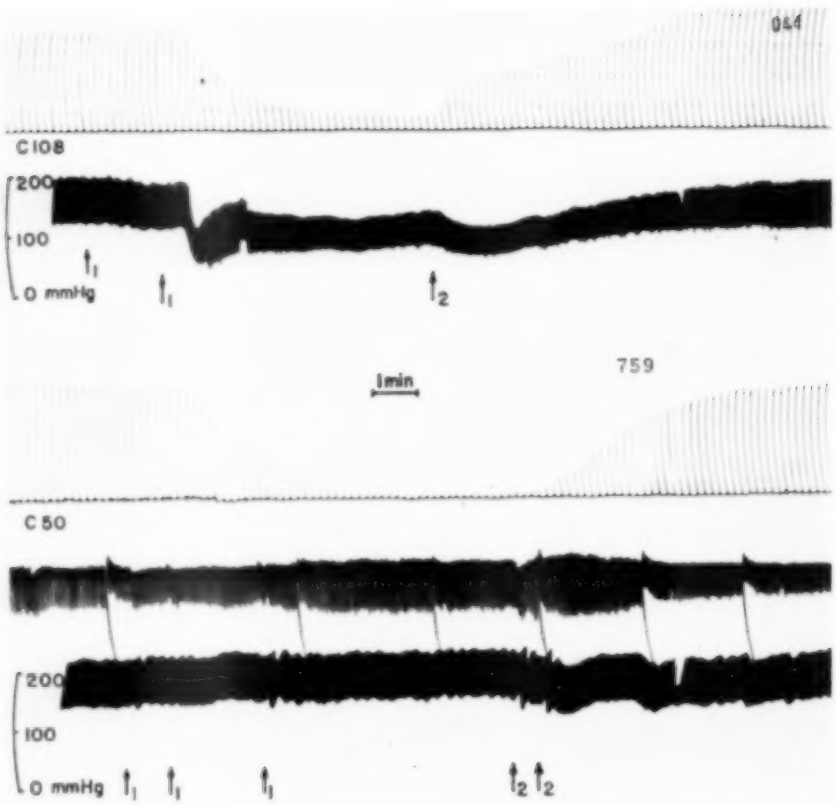


FIG. 2. *Top panel.* Cat 108, 3 kg. At each of arrows 1, 100 μg of *d*-tubocurarine per kg, intravenously. At arrow 2, thiamine, 10 mg/kg, intravenously. *Bottom panel.* Cat 50, 3·8 kg. At each of arrows 1, 5 μg of decamethonium per kg, intravenously. At each of arrows 2, 5 mg of thiamine per kg, intravenously.

Vol. 7
1961

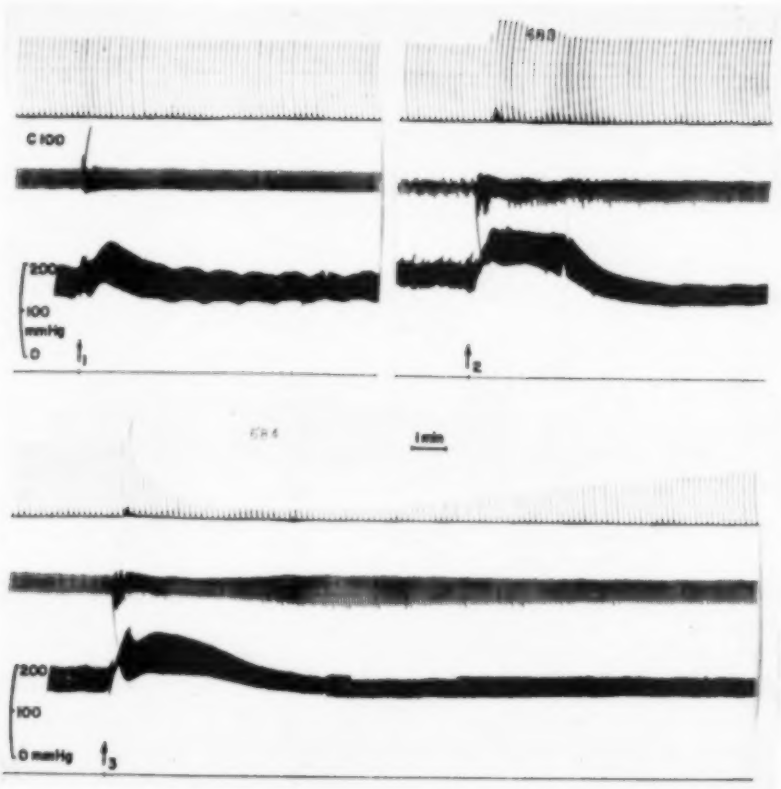


FIG. 3. Cat 100, 2·9 kg. At arrow 1, methyl thiazolium iodide, 2 mg/kg, intravenously; at arrow 2, 10 mg/kg. Note the increase in twitch response, respiratory rate and amplitude and arterial pressure. After 20 mg of the same compound per kg, injected at arrow 3, the brief initial increase in twitch was followed by partial paralysis.

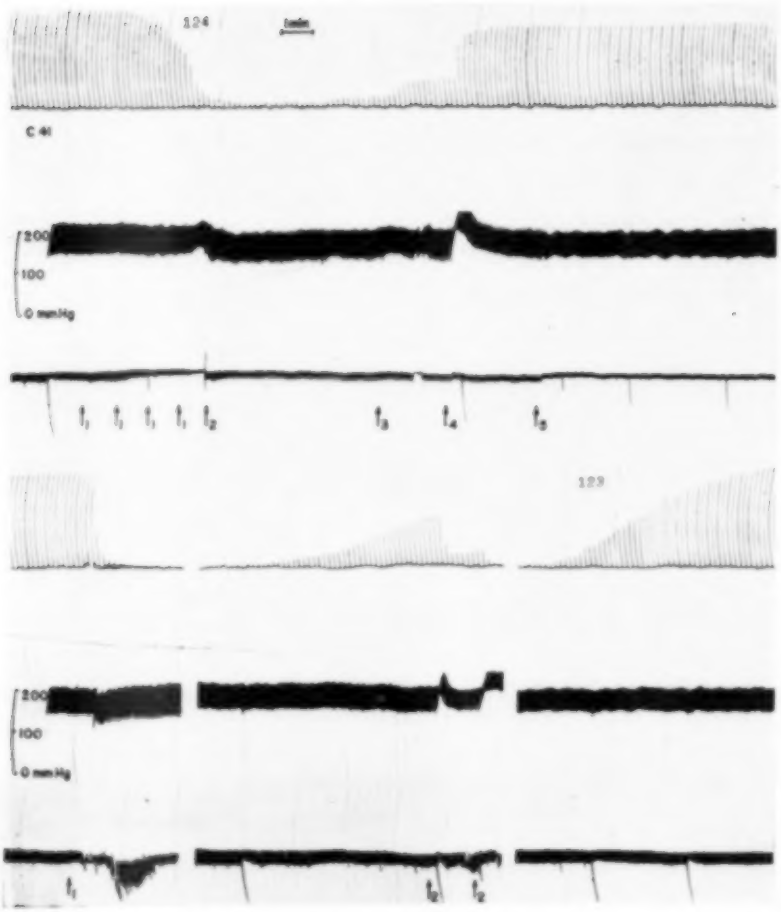


FIG. 4. Cat 41, 1·9 kg. *Top panel.* At each of arrows 1, 50 μg of *d*-tubocurarine per kg, intravenously. At arrow 2, started artificial respiration. At arrow 3, 2 mg and arrow 4, 5 mg, of methyl pyridinium iodide per kg, intravenously. The twitch response showed partial recovery after the first injection and complete recovery after the second. At arrow 5, artificial respiration was discontinued. *Bottom panels.* At arrow 1, 50 μg of decamethonium per kg, intravenously. Fifteen-minute interval between left and center panels. Artificial respiration employed during this period. At each of arrows 2, 5 mg of methyl pyridinium iodide per kg, intravenously. Note step-like decrease in the twitch. Artificial respiration was started shortly afterward. Fifty-minute interval between right and center panels.

C. *Thiamine and pyrithiamine analogs in which there is no hydroxy group on the thiazolium or pyridinium ring*

3-(4-Amino-2-methyl pyrimidyl-5-methyl)-thiazolium bromide, 1-(4-amino-2-methyl pyrimidyl-5-methyl)-pyridinium bromide, and 1-(4-amino-2-methyl pyrimidyl-5-methyl)-2-methyl pyridinium bromide.

1. *Effect on tibialis twitch response, respiration and arterial pressure.* 3-(4-Amino-2-methyl pyrimidyl-5-methyl)-thiazolium bromide (0·4–1 mg/kg), 1-(4-amino-2-methyl pyrimidyl-5-methyl)-pyridinium bromide (0·2–0·4 mg/kg) and 1-(4-amino-2-methyl pyrimidyl-5-methyl)-2-methyl pyridinium bromide (0·5–5 mg/kg) all increased twitch response. With larger doses, 2–3 mg/kg, 0·4–0·6 mg/kg and 5–20 mg/kg, respectively, the twitch response increased and this increase was accompanied by generalized muscular fasciculation; following these responses, the twitch decreased (Fig. 5).

With 3-(4-amino-2-methyl pyrimidyl-5-methyl)-thiazolium bromide, transient decrease in respiratory amplitude was followed by increase in respiratory rate and amplitude. There was a more than 50 mm Hg elevation of arterial pressure, lasting for 5–10 min. 1-(4-Amino-2-methyl pyrimidyl-5-methyl)-pyridinium bromide first increased the amplitude of respiration, and with larger doses, decreased it and finally produced apnea. This compound had no effect on the arterial pressure. 1-(Amino-2-methyl pyrimidyl-5-methyl)-2-methyl pyridinium bromide, at the dose levels studied (0·5–20 mg/kg), caused no significant change in respiration. The arterial pressure was was only slightly reduced.

2. *Effect on neuromuscular block produced by* d-*tubocurarine and decamethonium.* All three compounds, in doses varying from 1–5 mg/kg, antagonized *d*-tubocurarine and potentiated decamethonium (Fig. 6). However, the compound with a methyl group on the 2-position of the pyridinium ring was less potent in this respect than the other two. Also, with larger doses, the action of these compounds in antagonizing *d*-tubocurarine was transient. The transient increase in twitch response was followed by a marked potentiation of block.

D. *Pyrithiamine analogs in which there is a hydroxy group on the pyridinium ring at different positions*

1-(4-Amino-2-methyl pyrimidyl-5-methyl)-2-hydroxymethyl pyridinium bromide, -3-hydroxymethyl pyridinium bromide, -4-hydroxymethyl pyridinium bromide and -3-hydroxy pyridinium bromide.

1. *Effect on tibialis twitch response, respiration and arterial pressure.* Only one compound in this category, 1-(4-amino-2-methyl pyrimidyl-5-methyl)-3-hydroxymethyl pyridinium bromide, was tested for these actions. From 1 to 20 mg of this compound per kg did not significantly alter twitch response; 50 mg/kg caused a minimal decrease in twitch response. With doses of 10 mg or more per kg, there was a slight increase in respiratory rate and amplitude, and a moderate to marked decrease in arterial pressure.

2. *Effect on neuromuscular block produced by* d-*tubocurarine and decamethonium.* With the exception of 1-(4-amino-2-methyl pyrimidyl-5-methyl)-2-hydroxymethyl pyridinium bromide, all the compounds in this group (in doses of 5–10 mg/kg) antagonized both *d*-tubocurarine and decamethonium (Fig. 7). With decamethonium,

the antagonistic effect was comparable to that of thiamine. With *d*-tubocurarine, these compounds are less potent than thiamine. As shown in the bottom panel of Fig. 8, 1-(4-amino-2-methyl pyrimidyl-5-methyl)-3-hydroxymethyl pyridinium bromide, 5 mg/kg, increased the twitch response only slightly. The twitch remained at this new level with no further progressive recovery, and a second dose of the same compound had no effect.

1-(4-Amino-2-methyl pyrimidyl-5-methyl)-2-hydroxymethyl pyridinium bromide antagonized *d*-tubocurarine. When tested against decamethonium, the results were inconsistent. In four of six instances there was no apparent effect; transient potentiation was observed in the fifth; antagonism in the sixth.

DISCUSSION

The neuromuscular blocking action of thiamine as observed in this study is similar to that observed by Smith *et al.*,[3] Gjone,[4] Cheymol *et al.*[5] and di Palma and Hitchock.[6] The nature of the blockade appears to resemble that produced by *d*-tubocurarine. Nastuk and Kahn[8] found that in frog sciatic nerve–sartorius preparations, thiamine reduced the twitch response without initially potentiating it. The threshold concentration of acetylcholine required to activate the muscle was elevated (see also Torda and Wolff[1]). Membrane potential studies revealed that thiamine did not significantly alter the resting membrane potential but that a 2–3 millimolar solution of this compound reduced the amplitude, and decreased the rate of decay, of the end-plate potential; at the 4-millimolar level, thiamine blocked axonal conduction. Thus, it seems that thiamine possesses multiple actions, one of which is neuromuscular blockade through competitive inhibition.

It has been suggested that the neuromuscular blocking activity of thiamine resides in the thiazolium portion, which contains the quarternary nitrogen.[6] This seems indeed to be the case, as the pyrimidine fraction apparently has no effect on the tibialis twitch response. However, the action of methyl thiazolium iodide is distinctly different from that of thiamine. The results indicate that methyl thiazolium iodide, being an onium derivative, activates the neuromuscular junction and, in larger doses, produces neuromuscular blockage through depolarization. The addition of β-hydroxyethyl and methyl groups to thiazolium (3:4-dimethyl-5-β-hydroxyethyl thiazolium iodide) significantly reduces its potency (see above). This finding is in agreement with the thesis of Riker[9] that the addition of a hydroxy group to onium ions reduces their excitatory action at the neuromuscular junction.

The coupling of thiazolium with pyrimidine produces a compound which differs structurally from thiamine only by the absence of the β-hydroxethyl and methyl groups in the thiazolium ring. This coupling with pyrimidine increases the potency of thiazolium but does not qualitatively alter the nature of its activity at the neuromuscular junction, namely, depolarization.

Parallel results were obtained with methyl pyridinium iodide, 1-(4-amino-2-methyl pyrimidyl-5-methyl)-pyridinium bromide and pyrithiamine hydrobromide in respect to the nature of the neuromuscular blockade produced and their relative potency.

It appears, therefore, that the action of thiamine and pyrithiamine depends upon several components of their complex molecules. The quarternary nitrogen in the thiazolium or the pyridinium ring is the component which imparts the neuromuscular

Vol. 7
1961

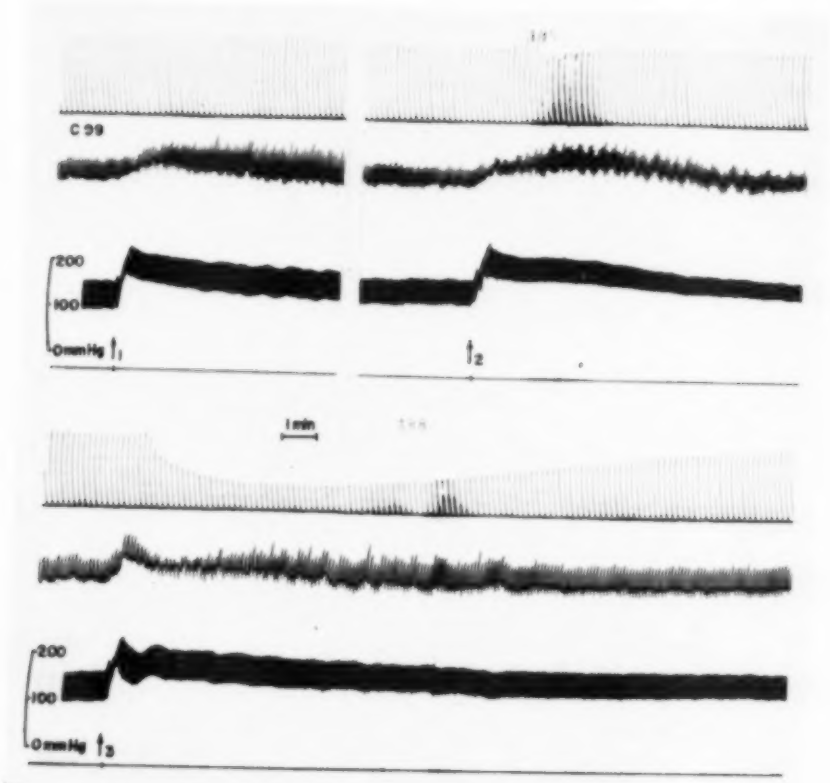


FIG. 5. Cat 99, 2·5 kg. Intravenous injections of 3-(4-amino-2-methyl pyrimidyl-5-methyl)-thiazolium bromide; at arrow 1, 0·6 mg/kg; at arrow 2, 1 mg/kg; and at arrow 3, 2 mg/kg. The effects on parameters measured were similar to those of methyl thiazolium iodide, as shown in Fig. 3.

facing page 262

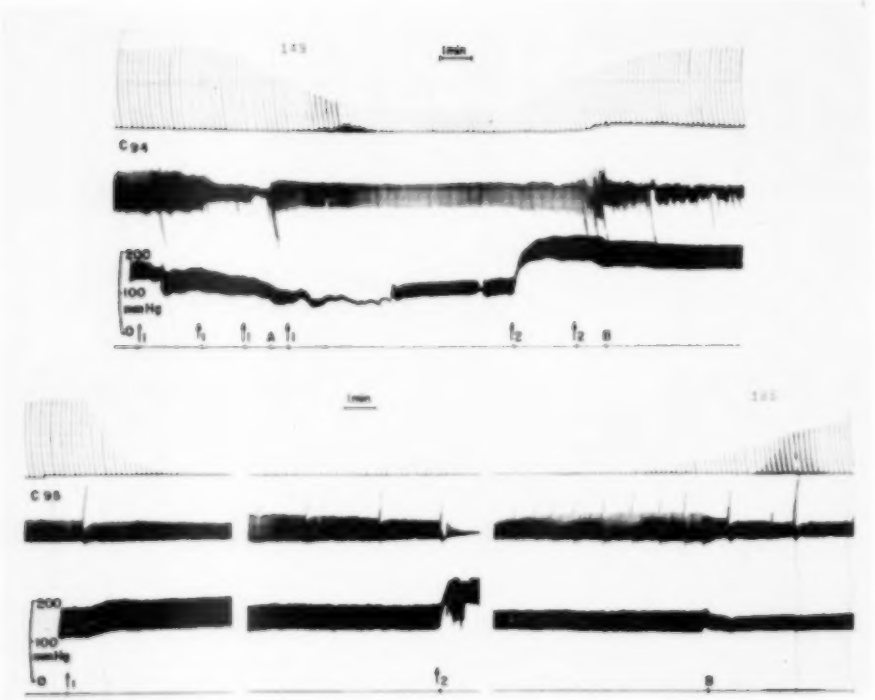


FIG. 6. *Top panel.* Cat 94, 2 kg. At each of arrows 1, 100 μg of *d*-tubocurarine per kg, intravenously. At each arrows 2, 2 mg of 3-(4-amino-2-methyl pyrimidyl-5-methyl)-thiazolium bromide per kg, intravenously. Between points *A* and *B*, artificial respiration. *Bottom panels.* Cat 95, 2·5 kg. At arrow 1, 20 μg of decamethonium per kg, intravenously. At arrow 2, 2 mg of 3-(4-amino-2-methyl pyrimidyl-5-methyl)-thiazolium bromide per kg, intravenously. Thirteen-minute interval between left and center panels; 17-min interval between center and right panels. Artificial respiration was started right after the center panel; stopped at point *B*.

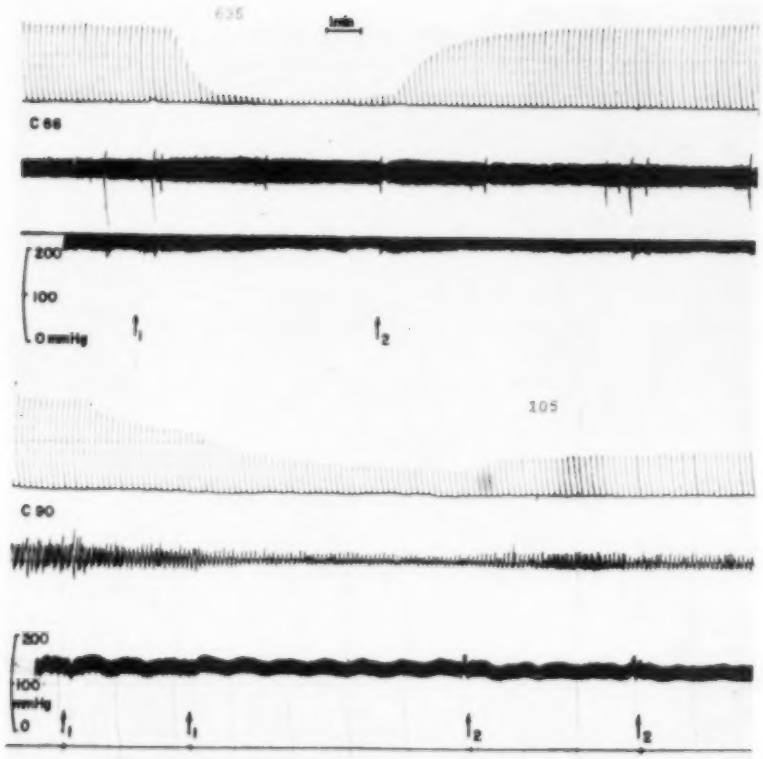


FIG. 7. *Top panel.* Cat 66, 2·5 kg. At arrow 1, 20 μg of decamethonium per kg, intravenously. At arrow 2, 5 mg of 1-(4-amino-2-methyl pyrimidyl-5-methyl)-3-hydroxymethyl pyridinium bromide per kg, intravenously. Twitch recovered gradually. No significant change in respiration and arterial pressure. *Bottom panel.* Cat 90, 2·5 kg. At each of arrows 1, 100 μg of *d*-tubocurarine per kg, intravenously. At each of arrows 2, 5 mg of 1-(4-amino-2-methyl pyrimidyl-5-methyl)-3-hydroxymethyl pyridinium bromide per kg, intravenously. Note the slight increase in twitch and an increase in respiratory amplitude after the first dose. Twitch stayed at the new level without further progressive recovery. The second dose was without effect.

BLANK
PAGE

Vol. 7
1961

junctional activity. Coupling of thiazolium or pyridinium with pyrimidine increases its potency. Further addition of a hydroxy group to the quarternary nitrogen-bearing ring radically modifies the nature of the junctional activity to resemble that of *d*-tubocurarine.

It is interesting that thiamine, pyrithiamine and pyrithiamine analogs *with* a hydroxy group on the pyridinium ring usually caused a fall in arterial pressure. This fall is probably due to their ganglionic blocking activity, as demonstrated by di Palma and Hitchcock[6] (thiamine and pyrithiamine). On the other hand, thiazolium, pyridinium and the "pyrimidyl" analog of thiazolium *without* the hydroxy group on the quarternary nitrogen-bearing ring always produced a marked elevation of arterial pressure. Although the mechanism of this action was not investigated in the present study, it is most likely that these compounds have a ganglionic stimulating action in addition to their neuromuscular junctional activity.

The influence of thiamine, pyrithiamine and their analogs on the neuromuscular block produced by *d*-tubocurarine and decamethonium is definitely related to their molecular structures. Thus, thiazolium, pyridinium and their "pyrimidyl" analogs *without* the hydroxy group on the quarternary nitrogen-bearing ring, always antagonized *d*-tubocurarine and potentiated decamethonium. Such effects of these compounds are not surprising, in view of the fact that they appear to have an excitatory action at the neuromuscular junction. Thiamine, pyrithiamine, oxythiamine and pyrithiamine analogs *with* a hydroxy group on the pyridinium ring, with one exception (see results), usually antagonized the action of both *d*-tubocurarine and decamethonium.

The antagonistic effect of this group of compounds on both types of neuromuscular block is interesting in that their own action resembles that of *d*-tubocurarine. Results from the present study provide no clue as to the mechanisms of this action. However, it would appear difficult to explain these findings within the context of the present concept of neuromuscular transmission and of the mode of action of *d*-tubocurarine and decamethonium. It should be pointed out that the antagonistic action of thiamine is evident only in *in vivo*-experiments. Thiamine potentiates the action of *d*-tubocurarine, decamethonium, and succinylcholine in rat diaphragm preparation *in vitro*.[4] Also, the antagonistic action of thiamine against *d*-tubocurarine is reversed with larger doses (10–15 mg/kg) or by its successive administration (see also Cheymol *et al.*[5]).

A few other compounds have been reported capable of antagonizing both *d*-tubocurarine and decamethonium. These are 1:5-bis-(allyldimethyl ammonium phenyl) penta-3-one dibromide (B.W. drug no. 53–67 or drug no. 284-C-51, an anti-acetylcholinesterase),[10, 11] N:N′-bis-(2-diethylaminoethyl)oxamide bis-2-methoxybenzyl chloride) WIN 8078,[12, 13] and tetraethylammonium chloride.[14–16] The molecular structures of these compounds are different from one another and distinct from that of thiamine and its analogs. Their mode of action at the neuromuscular junction and the mechanisms for their antagonistic action against the relaxants are probably also different. These problems remain to be investigated.

Acknowledgement—The authors are grateful for the valuable advice of Dr. Irwin B. Wilson.

REFERENCES

1. C. Torda and H. G. Wolff, *Proc. Soc. Exp. Biol., N.Y.* **56**, 89 (1944).
2. K. Unna and E. P. Pick, *J. Pharmacol.* **81**, 294 (1944).
3. J. A. Smith, P. P. Foa and H. R. Weinstein, *Science* **108**, 412 (1948).

4. E. GJONE, *Acta Pharmacol. Tox.* **11**, 55 (1955).
5. J. CHEYMOL, F. BOURILLET, C. LEVASSORT and L. KERP, *Arch. Int. Pharmacodyn.* **111**, 36 (1957).
6. J. R. DI PALMA and P. HITCHCOCK, *Anesthesiology* **19**, 762 (1958).
7. R. R. WILLIAMS, R. E. WATERMAN, J. C. KERESZTESY and E. R. BUCHMAN, *J. Amer. Chem. Soc.* **57**, 536 (1935).
8. W. L. NASTUK and N. KAHN, *Fed. Proc.* **19**, 260 (1960).
9. W. F. RIKER, JR., *Pharmacol. Rev.* **5**, 1 (1953).
10. C. H. ELLIS, A. L. WNUCK, S. NORTON and E. J. DEBEER, *Fed. Proc.* **13**, 40 (1954).
11. E. J. DEBEER, *Proceedings of the Conference on Myoneural Junction, New York City* p. 154 (1956).
12. A. G. KARCZMAR, *J. Pharmacol.* **119**, 39 (1957).
13. K. C. KIM, A. G. KARCZMAR and K. KOKETSU, *Fed. Proc.* **19**, 261 (1960).
14. C. J. KENSLER, *Brit. J. Pharmacol.* **5**, 204 (1950).
15. J. STOVNER, *Acta Anesth. Scand.* **2**, 53 (1958).
16. J. STOVNER, *Acta Anesth. Scand.* **2**, 165 (1958).

Vol.
196

Biochemical Pharmacology, 1961, Vol. 7, pp. 265–270. Pergamon Press Ltd., Printed in Great Britain.

THE METABOLISM OF DRUGS BY REGENERATING LIVER*†

JAMES R. FOUTS, ROBERT L. DIXON and ROBERT W. SHULTICE

Department of Pharmacology, College of Medicine,
State University of Iowa, Iowa City, Iowa

(*Received* 11 *March* 1961)

Abstract—Liver during and immediately following the rapid regeneration caused by partial hepatectomy is deficient in ability to oxidize the side-chain of hexobarbital, oxidize the ring-sulfur of chlorpromazine, and reduce the nitro-group of *p*-nitrobenzoic acid. Full recovery of these enzyme activities occurs at about the same time as complete regeneration of hepatic mass (approx. 10 days after operation). The rate of O-dealkylation of codeine is affected only during the first (and most rapid) phase of regeneration; following this period of rapid regeneration (approx. 4 days after operation), this enzyme activity is the same as in unoperated control animals.

The lower enzyme activities are not caused by a lack of known cofactors or by the presence of inhibitors.

Sham-operation affects the rates of metabolism of hexobarbital and chlorpromazine, but not those of codeine or *p*-nitrobenzoic acid. The effects of sham-operation are significantly less than those of partial hepatectomy.

WORK in our laboratory has shown that hepatic tumors[1] and livers from new-born animals[2] are deficient in ability to metabolize a variety of drugs. It appeared that hepatic cells dividing rapidly may be absolutely or relatively deficient in such enzyme activities. Therefore, we studied the metabolism of several drugs by the microsomal fraction prepared from regenerating liver.

Von der Decken and Hultin[3] have reported that the microsomal enzyme systems which demethylate and cleave *p*-monomethylaminoazobenzene (MAB) were less active than "normal" in livers from animals after partial hepatectomy.

We have investigated (a) whether regenerating liver metabolized only one or several drugs more slowly than normal liver, (b) the extent to which enzyme activities were depressed, (c) whether the changes seen were fully reversible, and if so, how quickly, and (d) whether decreases in activity might be caused by deficiencies in cofactor (reduced triphosphopyridine nucleotide (TPNH)), or the presence of inhibitors.

MATERIALS AND METHODS

Male Holtzman rats weighing 200–250 g were used. Partial hepatectomies were performed according to the method of Higgins and Anderson.[4] Sham-operated animals were anesthetized with ether, the peritoneum was opened, the liver exteriorized, and

* Supported by grants from the National Institutes of Health (RG 6034 and Training Grant 2G-141).

† The authors wish to acknowledge the work of Mr. Floyd Leaders, Mr. Larry G. Hart, Mrs. Anne Trankle, and Mrs. Donna Goodwin of our department, who helped with the hepatectomies and various assays.

the wound closed as in the hepatectomized animals. From seventy to eighty rats were operated on the same day. Every morning thereafter, control (unoperated), sham-operated, and hepatectomized rats were killed, the livers excised, and samples taken for histological examination by the Pathology Department*. Livers used in enzyme assays were chilled and homogenized with 2 vols of 1·15% KCl. The 9000 g supernatant fractions containing microsomal and soluble enzymes were prepared in the cold from homogenates using a high speed angle centrifuge.

The metabolisms studied were the side-chain oxidation of hexobarbital, the O-dealkylation of codeine, the ring-sulfur oxidation of chlorpromazine, and the reduction of the nitro-group of *p*-nitrobenzoic acid. Disappearance of substrate was measured in following the metabolisms of hexobarbital and chlorpromazine. The appearance of a metabolite (morphine and *p*-aminobenzoic acid) was measured to follow the metabolisms of codeine and *p*-nitrobenzoic acid, respectively. Hexobarbital was determined by the method of Brodie and Axelrod.[5] Chlorpromazine was assayed by the method of Salzman and Brodie.[6] The metabolism of *p*-nitrobenzoic acid was followed as reported previously.[7] Morphine was determined by using a phenol reagent[8] after extraction from tissue with chloroform. The incubation conditions used, cofactors added and their concentrations have been described.[9] Glucose-6-phosphate dehydrogenase was assayed by the method of Kornberg and Horecker.[10]

Duration of action of hexobarbital *in vivo* was assessed using "sleeping times" of rats injected intraperitoneally with hexobarbital sodium (100 mg/kg). The time during which the righting reflex was absent was called the sleeping time. No rat was used more than once in sleeping time determinations.

Statistical methods used are described by Snedecor.[11] We have chosen the value $P = 0{\cdot}05$ as the level of significance.

RESULTS

Tables 1, 2, 3 and 4 show that all drug metabolizing enzymes studied were present in livers regenerating after partial hepatectomy. From 48 to 192 hr after hepatectomy the metabolisms of hexobarbital, chlorpromazine, and *p*-nitrobenzoic acid were significantly slower than control.

The effect of sham-operation also is seen in Tables 1 and 2. Sham-operation affected the metabolisms of hexobarbital and chlorpromazine. At 72, 120, 168 and 192 hr after sham-operation the rates of metabolism of codeine and *p*-nitrobenzoic acid were not significantly different from control. At times of maximum depression, drug-metabolizing enzyme activities in hepatectomized animals were significantly less than those in sham-operated animals. Enzyme activity in regenerating liver leveled off at a minimum, for all metabolisms except codeine, between 72 and 192 hr after hepatectomy. The degree of depression of metabolism (expressed as per cent of control) was not the same for all pathways. Most affected was the metabolism of *p*-nitrobenzoic acid. Least depressed was the O-dealkylation of codeine. The rate of metabolism of codeine by livers from hepatectomized animals differed from the control rate only at 48 and 72 hr after operation. At all other times there was no significant difference in the rate of this metabolism between hepatectomized and control animals.

* We also wish to thank Dr. Fernando Aleu of the Pathology Department for his help in the many histological examinations.

Vol. 1
1961

TABLE 1. METABOLISM OF CHLORPROMAZINE BY 9000 g SUPERNATANT FRACTIONS OF RAT LIVER

Hours after operation	Metabolism by §						
	Unoperated (control) (μmoles*/g liver)	Hepatectomized (μmoles*/g liver)	P†	% of control activity	Sham-operated (μmoles*/g liver)	P‡	% of control activity
0	1·1 ± 0·28	—	—	—	—	—	—
48		0·63 ± 0·22	0·005	57	0·60 ± 0·19	>0·5	54
72		0·45 ± 0·28	<0·001	41	0·85 ± 0·27	0·025	77
96		0·35 ± 0·31	<0·001	32	1·3 ± 0·51	<0·001	118
120		0·42 ± 0·31	<0·001	38	1·2 ± 0·22	0·005	109
144		0·47 ± 0·24	<0·001	43	1·0 ± 0·43	0·010	91
168		0·32 ± 0·24	<0·001	29	0·64 ± 0·22	0·05	58
216		0·79 ± 0·17	0·025	72	1·3 ± 0·30	0·005	118
288		1·5 ± 0·10	<0·001	136	1·4 ± 0·26	0·2	127

* Values are average μmoles drug metabolized ± standard deviation per g liver (wet weight).

† Probability that there is no difference between the means of hepatectomized vs. control groups other than that caused by random error in measurement. Student *t*-test.

‡ Probability that there is no difference between the means of hepatectomized vs. sham-operated groups.

§ All groups had seven animals except control which had fourteen.

TABLE 2. METABOLISM OF HEXOBARBITAL BY 9000 g SUPERNATANT FRACTIONS OF RAT LIVER

Hours after operation	Unoperated (control) (μmoles*/g liver)	Hepatectomized (μmoles*/g liver)	n§	P†	% of control activity	Sham-operated (μmoles*/g liver)	n§	P‡	% of control activity
0	4·1 ± 1·2	—		—	—	—		—	—
48		1·7 ± 0·5	(15)	<0·001	41	2·5 ± 0·7	(7)	0·025	61
72		1·6 ± 0·5	(10)	<0·001	39	3·0 ± 0·8	(7)	<0·001	73
96		1·6 ± 0·6	(12)	<0·001	39	2·6 ± 0·4	(7)	<0·001	63
120		1·5 ± 0·6	(10)	<0·001	37	3·3 ± 0·8	(7)	<0·001	80
144		1·6 ± 1·1	(11)	<0·001	38	2·9 ± 0·6	(7)	0·005	71
168		1·5 ± 0·7	(11)	<0·001	37	3·0 ± 0·7	(7)	<0·001	73
192		1·4 ± 0·1	(8)	<0·001	34	—		—	—
216		2·3 ± 0·8	(15)	<0·001	57	2·7 ± 0·8	(7)	0·4	66
288		3·9 ± 0·6	(7)	>0·5	95	2·8 ± 0·3	(7)	0·001	68

* Values are average μmoles drug metabolized ± standard deviation per g liver (wet weight).

† Probability that there is no difference between the means of hepatectomized vs. control groups other than that caused by random error in measurement. Student *t*-test.

‡ Probability that there is no difference between the means of hepatectomized vs. sham-operated groups.

§ *n* = number of animals per group. Control groups had fifteen animals.

Full recovery of enzyme activities for the metabolism of hexobarbital, chlorpromazine, and *p*-nitrobenzoic acid occurred between 216 and 288 hr. This was at approximately the time when complete regeneration of hepatic mass has been reported to occur.[4] The rate of metabolism of codeine returned to control levels when the first phase of rapid regeneration had stopped—reported by Higgins and Anderson to occur 3 to 4 days after hepatectomy.[4]

Nicotinamide, TPN, $MgSO_4$, and glucose-6-phosphate were added in every assay. Glucose-6-phosphate dehydrogenase activity (generates TPNH) in livers from hepatectomized animals was at all times equal to or greater than control. As far as we could determine, the lower rates of metabolism of drugs *in vitro* were not caused by a deficiency of TPNH, the only known cofactor of these systems.

TABLE 3. METABOLISM OF CODEINE BY 9000 g SUPERNATANT FRACTIONS OF RAT LIVER

Hours after operation	Unoperated (control) (μmoles*/g liver)	Hepatectomized (μmoles*/g liver)	n‡	P†	% of control activity
0	1·6 ± 0·4	—	—	—	—
48		1·1 ± 0·3	(15)	0·005	69
72		1·2 ± 0·3	(15)	0·025	75
96		1·4 ± 0·4	(15)	0·2	88
120		1·3 ± 0·5	(11)	0·2	81
144		1·7 ± 0·4	(11)	0·4	106
168		1·5 ± 0·2	(11)	>0·5	94
192		1·4 ± 0·7	(4)	>0·5	88
216		1·8 ± 0·3	(11)	0·5	113

* Values are average μmoles drug metabolized ± standard deviation per g liver (wet weight).
† Proability that there is no difference between the means of hepatectomized vs. control groups other than that caused by random error in measurement. Student *t*-test.
‡ *n* = number of animals per group. Control group had fifteen animals.

TABLE 4. METABOLISM OF *p*-NITROBENZOIC ACID BY 9000 g SUPERNATANT FRACTIONS OF RAT LIVER

Hours after operation	Unoperated (control) (μmoles*/g liver)	Hepatectomized (μmoles*/g liver)	n‡	P†	% of control activity
0	1·7 ± 0·2	—	—	—	—
48		0·80 ± 0·09	(8)	<0·001	47
72		0·78 ± 0·17	(8)	<0·001	46
96		0·30 ± 0·10	(7)	<0·001	18
120		0·25 ± 0·01	(8)	<0·001	15
144		0·59 ± 0·12	(8)	<0·001	35
168		0·41 ± 0·01	(8)	<0·001	24
192		0·61 ± 0·12	(4)	<0·001	36
216		1·1 ± 0·17	(8)	<0·001	65
288		2·0 ± 0·22	(7)	0·025	116

* Values are average μmoles drug metabolized ± standard deviation per g liver (wet weight).
† Probability that there is no difference between the means of hepatectomized vs. control groups other than that caused by random error in measurement. Student *t*-test.
‡ *n* = number of animals per group. Control group had eight animals.

Mixtures of 9000 g supernatant fractions from control rats and from hepatectomized rats metabolized drugs at rates which were not less than those of the control supernatant fraction alone It appeared that there were no enzyme inhibitors in livers from hepatectomized animals.

The duration of action of hexobarbital was used to estimate the rate of metabolism of this drug *in vivo*. Hepatectomized rats slept longer after a given dose of hexobarbital than did control or sham-operated animals. Table 5 shows that a prolonged sleeping

Vol. 7
1961

time was found at all times when the *in vitro*-assays showed the rate of hexobarbital metabolism to be less than in sham-operated animals. The depression *in vitro* of the metabolism of hexobarbital that was seen in sham-operated animals was not sufficient to affect the sleeping time *in vivo*. Thus, the duration of action of hexobarbital in sham-operated animals was not significantly different from that in control animals (Table 5).

TABLE 5. THE DURATION OF ACTION OF HEXOARBITAL IN CONTROL AND HEPATECTOMIZED RATS

Hours after operation	Sleeping times				
	Unoperated (controls) (min)	Sham-operation (min)	n*	Hepatectomized (min)	n*
0	22 ± 6	—	—	—	—
72		26 ± 4	4	283 ± 69	4
96		22 ± 2	5	204 ± 68	5
120		31 ± 6	5	88 ± 14	6
144		21 ± 5	6	80 ± 12	5
168		24 ± 4	5	85 ± 15	6
192		24 ± 6	5	54 ± 8	7
216		—	—	48 ± 8	6
240		—	—	50 ± 7	7
264		22 ± 5	5	42 ± 14	6
288		21 ± 3	5	27 ± 5	5

* n = number of animals in each group. Control group had thirty-two animals.

Histological examinations of sections of livers at all times after hepatectomy revealed no pathological changes which might be a cause of the lower enzyme activities.

DISCUSSION

Rapidly growing liver can be deficient in a number of microsomal enzymes which metabolize drugs. The pathways of metabolism affected and the extent of this effect depend upon the conditions causing rapid growth. In hepatic tumors[1] and in livers from newborn animals[2] the ability to metabolize hexobarbital, chlorpromazine, and *p*-nitrobenzoic acid is essentially absent. In regenerating liver after partial hepatectomy all of these metabolic activities are present, but their rates are less than control values. Between 48 and 192 hr after hepatectomy the rate of metabolism of *p*-nitrobenzoic acid is markedly depressed, while the rates of metabolism of hexobarbital and chlorpromazine also are depressed, but to a lesser extent.

Between 48 and 96 hr after partial hepatectomy, the rate of metabolism of codeine is less than in control animals This rate of metabolism is the same as in control animals from 96 to 216 hr after operation. Of all the drug metabolizing enzymes studied, only that for the metabolism of codeine returns to control levels of activity following the first phase of rapid regeneration (72–96 hr)

These results serve to emphasize again that not all drug-metabolizing enzymes are affected equally by the same conditions We have reported similar findings in studies on the metabolism of drugs in jaundiced rabbits,[9] starved mice[12] and diabetic rats.[13] von der Decken and Hultin[3] have shown that some microsomal enzyme activities were depressed after hepatectomy, while other activities were unchanged or elevated.

Their studies, showing a decrease in the rate of metabolism of MAB, are similar to our results, which show a decrease in the rate of metabolism of hexobarbital, chlorpromazine, and *p*-nitrobenzoic acid. However, one cannot generalize and say that all microsomal drug-metabolizing enzyme activities are depressed during the regeneration following hepatectomy.

The decrease in enzyme activities is unlikely to be caused by a deficiency of TPNH or the presence of enzyme inhibitors in regenerating liver At this time we cannot rule out a change in amount of active enzyme as the cause of the decreased activity.

The effects of sham-operation on the metabolism of drugs are not easily understood. It is apparent that all pathways are not affected by such operation. The rates of metabolism of hexobarbital and chlorpromazine are depressed, while those of codeine or *p*-nitrobenzoic acid are not affected. The effects of hepatectomy on the metabolism of hexobarbital and chlorpromazine are significantly greater than those of sham-operation at the times of the maximal effects of hepatectomy. The conclusions, though complicated by these effects of sham-operation, are still valid: the liver during and immediately after rapid regeneration is relatively deficient in its ability to metabolize certain drugs.

From such information as we have, one might speculate about the effects of certain hepatic injuries on the metabolism of drugs. In many cases, injury to the liver is followed by a period of regeneration. If such regeneration were extensive, the metabolism of drugs might be affected. Such effects on drug-metabolizing enzymes might not be predicted by presently used function tests or histological examinations, since these tests do not directly assess the status of the endoplasmic reticulum (or the microsomes which are derived from this reticulum).

Vol. 1
1961

REFERENCES

1. R. H. ADAMSON and J. R. FOUTS, *Cancer Res.* In press (1961).
2. J. R. FOUTS and R. H. ADAMSON, *Science* **129**, 897 (1959).
3. A. VON DER DECKEN and T. HULTIN, *Exp. Cell Res.* **19**, 591 (1960).
4. G. M. HIGGINS and R. M. ANDERSON, *Arch. Path.* **12**, 186 (1931).
5. B. B. BRODIE and J. AXELROD, *J. Pharmacol.* **99**, 171 (1950).
6. N. P. SALZMAN and B. B. BRODIE, *J. Pharmacol.* **118**, 46 (1956).
7. J. R. FOUTS and B. B. BRODIE, *J. Pharmacol.* **119**, 197 (1957).
8. F. D. SNELL and C. T. SNELL, *Colorimetric Methods of Analysis* p. 510. D. Van Nostrand, New York (1937).
9. E. F. MCLUEN and J. R. FOUTS, *J. Pharmacol.* **131**, 7 (1961).
10. A. KORNBERG and B. L. HORECKER, *Methods in Enzymology* (Edit. by S. P. COLOWICK and N. O. KAPLAN) Vol. I, p. 323. Academic Press, New York (1955).
11. G. W. SNEDECOR, *Statistical Methods*. Iowa State College Press, Ames, Iowa (1956).
12. R. L. DIXON, R. W. SHULTICE and J. R. FOUTS, *Proc. Soc. Exp. Biol., N.Y.* **103**, 333 (1960).
13. R. L. DIXON, L. G. HART and J. R. FOUTS, *J. Pharmacol.* In press.

Biochemical Pharmacology, 1961, Vol. 7, pp. 271–276. Pergamon Press Ltd., Printed in Great Britain.

BLOOD LEVELS AND CLEARING ACTIVITY OF A SYNTHETIC HEPARINOID

L. Levy and G. E. Cronheim

Riker Laboratories, Inc., Northridge, Calif.

(*Received* 16 *February* 1961)

Abstract—A new synthetic heparinoid, sulfopolyglucin (SPG), is compared with heparin with regard to clearing activity and anticoagulant activity. The anticoagulant activity of SPG follows a curve similar to blood radioactivity and metachromasia measurements. SPG differs from heparin in time of appearance and duration of elevated clearing factor levels in the blood. Some theoretical aspects of the clearing factor differences are discussed.

In recent years it has been found that heparin as well as many heparinoids are capable of clearing the plasma of visible lipemia in man and in animals.[1] This clearing action has been attributed to an enzyme called "clearing factor" or "lipoprotein lipase". It is believed that this enzyme is normally present in the blood in small amounts, but that upon injection of heparin or heparinoids, the amount in the blood increases many-fold. Considerable work by many investigators has gone into characterizing this enzyme, but at the present time little is known biochemically aside from some requirements for its activity and its localization in tissue.

Sulfopolyglucin (SPG) is a sulfated polysaccharide of 5–15 glucose units connected by α-1:4- and α-1:6-linkages with up to 3 sulfate groups per glucose unit. The SPG discussed in this paper has approximately 2·5 sulfate units per glucose residue. SPG resembles herapin in that both compounds have anticoagulant and lipemia-clearing activity.[2] However, quantitative differences exist in the activity and the metabolism of these two substances, as will be shown in this paper.

METHODS

Mongrel dogs were used throughout, and at least four dogs were used for each dose reported. All injections of heparin sodium USP (Lipo-Hepin Riker, 140 USP units/mg) and of SPG, as the potassium salt, were given intravenously, unless specifically noted. Radioactive SPG containing ^{35}S and non-radioactive SPG (21 USP anticoagulant units/mg) were synthesized in this laboratory and had a specific activity between 10^5 and 10^6 counts/min per mg. Plasma samples used for radioactivity measurements were either directly plated or digested with Pirie's reagent, precipitated as $BaSO_4$, and then plated. All counts were made on a Nuclear Chicago Gas Flow Counter at infinite thinness. The injection solutions were treated for radioactive measurements in the same way as the blood and urine samples, in order to make valid comparisons. Clearing activity was measured by a modified Grossman procedure using Ediol as the substrate.[2] Anticoagulant activity was measured by the Lee–White whole blood-clotting time. The spectrophotometric measurement of SPG used a modified Jaques

metachromasia procedure.[3] Ascending chromatography was used with water–ethanol–ammonium hydroxide (54:45:1) as the solvent. The chromatograms were developed with Azure A or sodium rhodizinate. These chromatograms were subsequently placed in cassettes with Kodak no screen X-ray film and developed according to conventional methods.

RESULTS

In order to determine how rapidly SPG left the blood, radioactive material was injected intravenously into dogs and blood samples were taken periodically for various measurements. In Fig. 1 is presented the results of a group of experiments using dogs (anesthetized with pentobarbital) which were given saline infusions, in order that there would be a urine flow of at least 1 ml/min. Ten milligrams of radioactive SPG per kg and all other doses (1, 3 and 5 mg/kg) given in this series of experiments had a

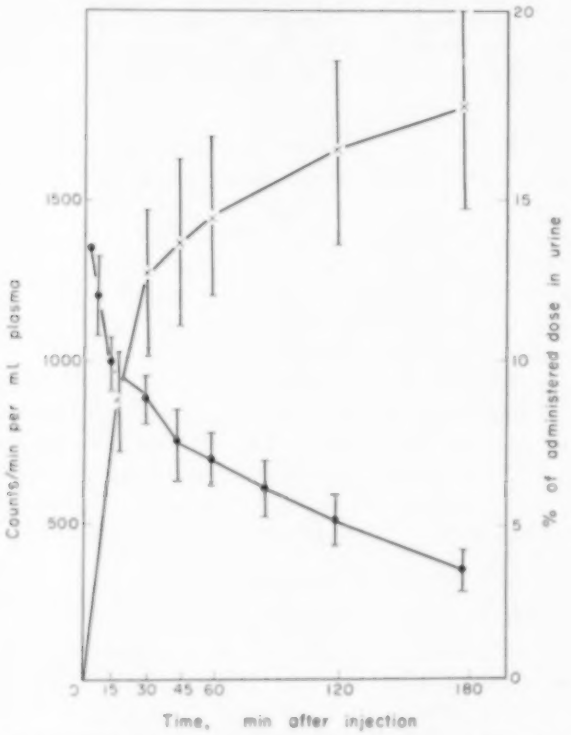


FIG. 1. These curves represent the mean (±s.e.) of at least four dogs after a single intravenous injection of 10 mg of radioactive SPG per kg. The percentage of the injected dose which appeared in the urine is represented by ×——×, while the counts/min per ml of plasma is represented by ●——●. The radioactivity in the plasma was 61 per cent of the total radioactivity of whole blood.

similar time-course in the blood, in that an initial rapid drop was followed by a slower fall. If the first and more rapid fall is a reflection of the dilution of the injected SPG by body fluids, then the slope of the second curve indicates either excretion or tissue distribution, or both. The urinary excretion curves in these same acute experiments

appear to be reciprocals of the blood levels (Fig. 1). On the basis that the second slope seen in Fig. 1 indicates a biological half-life of SPG in the blood of about 120 min, one would not expect to find measureable radioactivity in that tissue at the end of 12 hr or six biological half-lives. This has been verified repeatedly in that there has never been any radioactivity in the blood 12 hr after the injection, regardless of the size of the dose. According to the literature,[4] the decay curve for radioactive heparin is almost exactly the same as that presented in Fig. 1 for SPG. Loomis,[5] using radioactive heparin from the dog, found about 20 per cent of the radioactivity of the intravenously administered dose in the urine in 2 hr. The blood radioactivity curve of Loomis was also similar to that reported in this paper for SPG.

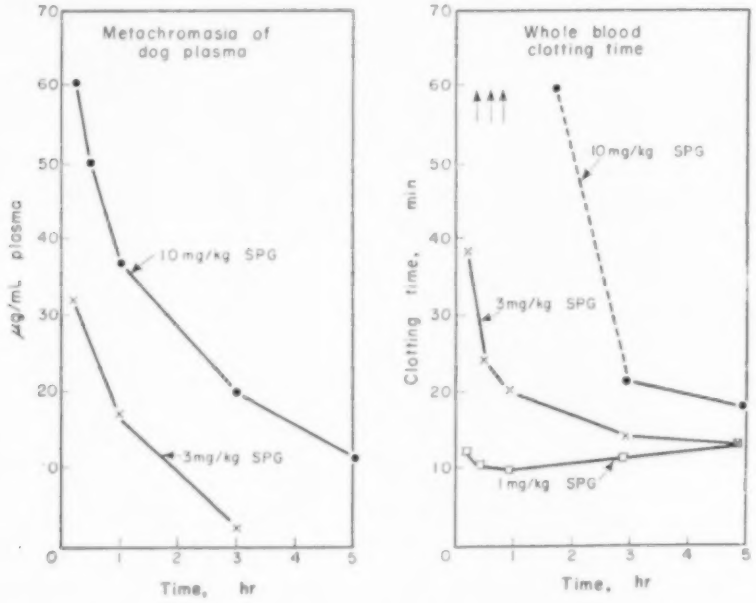


FIG. 2. These curves represent the mean value of at least four dogs. For additional information see text.

The experiments depicted in Fig. 1 do not give a good indication of whether the whole molecule or only the radioactive portion (sulfate residues) disappears from the blood. In order to determine whether part of the molecule was hydrolysed, metachromasia and anticoagulant activity were used as additional measurements. In Fig. 2, curves for both these parameters are shown; the data upon which these curves were based were derived from another set of dogs. It is apparent that the shape, as well as time-characteristics, of the two curves in Fig. 2 are similar to each other, as well as to the blood radioactivity curves seen in Fig. 1. In addition, anticoagulant time-curves for heparin USP had the same general shape and time characteristics as those for SPG, although the heparin had, on a weight basis, about seven times the anticoagulant potency of SPG.

Since, so far, all measurements of disposition showed a similarity between SPG and heparin, the slopes of the time-effect curves for lipemia-clearing activity (Fig. 3) were unexpected. For both doses illustrated here (2 and 5 mg/kg), heparin and SPG reached the same maximum activity, but the elevated levels for SPG were much more prolonged. The heparin peak occurred during the first hour, while the SPG peak was reached after about 3 hr. When higher dosages of these compounds were used, the peak activity for heparin was still early, but considerably elevated, while the peak activity of SPG was elevated to a lesser extent, but was reached with still greater delay.[2]

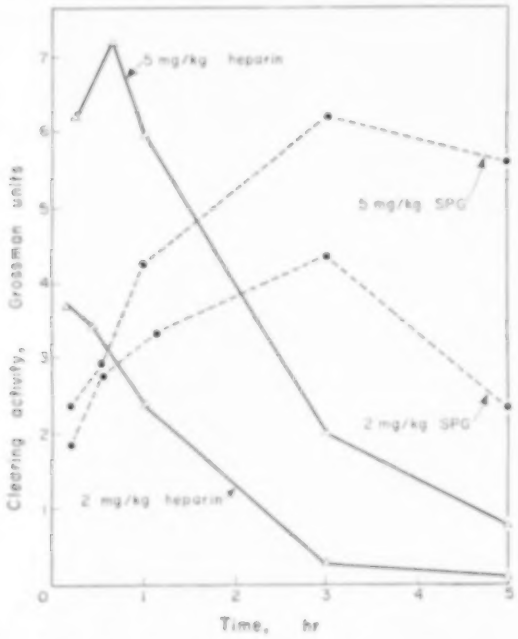


Fig. 3. These two curves show the average appearance time of lipoprotein lipase in the blood after a single intravenous injection of either SPG ●——● or heparin △——△.

Chromatograms of the urine of dogs receiving radioactive SPG, which were subsequently made into radioautographs, showed only the excretion of SPG. Development of duplicate chromatograms, using Azure A for identification of metachromatic material and rhodizinate for identification of inorganic sulfate, revealed the rapid appearance of only metachromatic material in the urine. Within 15 min after injection of an intravenous dose of SPG-^{35}S, a single metachromatic stain was found on the chromatogram which, when exposed to X-ray film, was also radioactive. After the injection of ^{35}S-heparin,[6] following which large amounts of ^{35}S-inorganic sulfate were found in the urine, no radioactive material differing from heparin was found on any of our films.

DISCUSSION

Korn[7] has proposed that heparin and heparin-like compounds are an essential part of the clearing factor or lipoprotein lipase molecule. The evidence presented in

this paper that the clearing factor curves for SPG and heparin are different in their temporal characteristics, is by itself not inconsistent with Korn's hypothesis, but the disappearance and excretion curves make this hypothesis unlikely.

If there is acquisition of the SPG molecule by the lipoprotein lipase, it would not be expected that the radioactivity of the blood would decrease while the level of enzyme activity in the blood increased. However, it is possible that the amount of radioactivity associated with the increase of lipoprotein lipase is too small to alter the measurements. The aforementioned possibility, in addition to evidence obtained by Robinson[8] and Korn[7] that samples of plasma from animals treated with heparin or dextran sulfate could be made to lose their lipoprotein lipase activity *in vitro* and *in vivo* with protamine or heparinase, make the combination of heparinoid with enzyme possible. However, the level of endogenous clearing factor in plasma is very low, and it seems more important to direct attention to the appearance of the enzyme in blood. Thus, it is more attractive to consider that heparin and other highly anionic substances cause either a release of clearing factor from the sites of storage or its increased synthesis, or both. Lipoprotein lipase has been found in various tissues, such as the heart and adipose tissue, and it is also believed to be in the vascular walls, as well as in other tissues.[1] Since SPG is chemically different from heparin, in that it is more acid, contains only glucose units, and has a lower molecular weight, it is possible that some of the primary sites for the release of clearing factor are affected by heparin and not by SPG. This would be consistent with other data which indicate that increasing intravenous doses of heparin only raise the peak-level of clearing activity, but do not extend the time-course, while higher intravenous doses of SPG not only raise the peak-level of clearing activity, but also extend the duration of elevated clearing activity in the blood. If, as an example, the sites in the vessel walls were the primary ones affected by heparin, while the sites in fat tissue were primarily affected by SPG, then the differences would be more readily understood.

Another possible factor to consider, in attempting to account for the differences in time-curves, is the rate of destruction of clearing factor. It has been suggested that the site of destruction of clearing factor *in vivo* is the liver. Since heparin and heparinoids have a great affinity for the liver,[4, 10–12] the action of SPG as well as heparin could cause alterations in the normal rate of destruction of clearing factor. Thus information consistent with this idea is derived from the observation that animals repeatedly given the same dose of SPG during long periods of time, tend to have higher and longer-lasting clearing activity in their blood than would be expected from the less frequent administration at similar amounts.[2] Since this cumulative drug action does not appear when heparin is administered repeatedly, the differences in the clearing activity curves might be explained by the action of SPG on the disposal mechanism for clearing factor in the liver and perhaps elsewhere.

It is possible that still other mechanisms not mentioned here may operate. However, it is felt that either of the above hypotheses, alone or together, could explain the difference between heparin and SPG. Experiments are in progress to clarify the similarities and differences between heparin and SPG.

Acknowledgement—The authors wish to thank M. Draper for synthesizing the radioactive compounds used in these experiments. We wish also to acknowledge the technical assistance of C. De Fusco and T. Hooper.

REFERENCES

1. D. S. Robinson and J. E. French, *Pharm. Rev.* **12**, 241 (1960).
2. E. Windsor and G. E. Cronheim, *Symposium on Thrombosis and Anticoagulant Therapy, Dundee, Scotland* (1960). In press.
3. L. B. Jaques and H. S. Bell, *Meth. Biochem. Anal.* **7**, 292 (1959).
4. H. B. Eiber, I. Danishefsky and F. S. Borelli, *Proc. Soc. Exp. Biol., N.Y.* **98**, 672 (1958).
5. T. A. Loomis, *Proc. Soc. Exp. Biol., N.Y.* In press.
6. I. Danishefsky and H. B. Eiber, *Arch. Biochem. Biophys.* **85**, 53 (1959).
7. E. D. Korn, *J. Biol. Chem*, **226**, 827 (1957).
8. D. S. Robinson, P. M. Harris and C. R. Ricketts, *Biochem. J.* **71**, 286 (1959).
9. H. Scholl and G. Schletter, *German Med. Mthly* **3**, 13 (1958).
10. P. E. Morrow, H. C. Hodge, W. F. Neuman, E. A. Maynard, H. S. Blanchet, Sr., D. W. Fassett, R. E. Birk and S. Manrodt, *J. Pharmacol.* **105**, 273 (1952).
11. E. Hausemann, E. G. Hoffman, R. Litterle and M. Wiedersheim, *Experientia* **8**, 153 (1952).
12. H. Weigel and K. Walton, *Nature, London* **183**, 981 (1959).

Vol. 7
1961

Biochemical Pharmacology, 1961, Vol. 7, pp. 277–279. Pergamon Press Ltd., Printed in Great Britain.

SHORT COMMUNICATIONS

The effect of thiamine on monoamine oxidase

(*Received* 17 *April* 1961)

IN ADDITION to the more widely studied inhibitors of monoamine oxidase (MAO), a number of quaternary nitrogen compounds inhibit this enzyme *in vitro*.[1] The structure of these compounds is very diverse, including long chain N-alkyl derivatives of pyridine, such as pyridine aldoxime dodecyliodide,[2] and ammonium salts such as hexamethonium and decamethonium.[1] In a recent publication,[3] Gal and Drewes demonstrated that in thiamine-deficient rats, the MAO-activity of the brain and intestine is increased, as compared to the activity of rats fed *ad libitum* with a normal laboratory diet. These workers suggested that the increase might be related to the stress theory of Selye. It is the purpose of this communication to propose that, *in vivo*, thiamine, which has a quaternary nitrogen in its thiazole moiety, is either itself an inhibitor of MAO, or more likely, is metabolized via a pathway which produces another quaternary nitrogen compound which is an inhibitor of MAO. Thus, the increased MAO-activity in the thiamine-deficient animals might be accounted for by a diminution in the amount of this thiamine-like inhibitor. The MAO-inhibition caused by this thiamine-like compound also might explain the hypotension and ganglionic blockade which thiamine produces in man and experimental animals;[4, 5] Gertner has recently shown that MAO-inhibitors block ganglionic transmission in cats and dogs,[6] while hypotension in man has been noted with the administration of inhibitors of MAO.[7]

Experiments *in vitro* show that thiamine and thiamine pyrophosphate are weak inhibitors of MAO: At 10^{-2} M, thiamine inhibits the MAO of rat liver mitochondria 70 per cent, as determined by the method of Weissbach *et al.*,[8] a result of little significance with respect to the proposed mechanism *in vivo*. Furthermore, rats given 70 mg of thiamine per kg subcutaneously, and sacrificed after 2 hr, when thiamine pyrosphosphate levels in liver are elevated,[9] showed no inhibition of the MAO-activity of liver, brain, and intestine, as compared with untreated controls. However, this does not constitute a refutation of the proposed theory, if the rate of formation of the postulated thiamine metabolite proceeds maximally with the amount of thiamine provided by a normal diet.

Substantial indirect evidence can be provided for the theory. Although in higher animals most of the thiamine which is absorbed is excreted unchanged, the fate of the thiamine which is degraded is largely unknown.[10] Molluscs, carp, and some other lower species possess an enzyme, thiaminase,[11] which catalyses the reaction of thiamine with a variety of basic compounds with loss of the 4-methyl-5-β-hydroxyethylthiazole portion, and the formation of other quaternary compounds derived from the pyrimidine portion.[10] No similar enzyme has been found in mammalian tissue, but only small amounts may be present. Such a reaction could produce a potent quaternary inhibitor of MAO. It is significant that von Muralt, using labeled thiamine, has shown that, on excitation of a peripheral nerve, metabolites of thiamine appear, which are as yet unidentified.[12] One of these might be the postulated inhibitor; if so, this would provide a mechanism for preventing the oxidation of any neurohumor which is a substrate for MAO.

The hypotension and ganglionic blockade produced both by thiamine and by known inhibitors of MAO is suggestive that these compounds are related. This action of thiamine is unrelated to its properties as a coenzyme.[4] The blockade is also different in kind from that produced by hexamethonium,[5] as is that caused by the inhibitors of monoamine oxidase.[6]

A final argument which may be offered is that in a thiamine-deficiency state in man (beri-beri), the initial symptoms are emotional—depression, apathy, and disinterest,[13, 14] a syndrome a physician might now treat with an inhibitor of MAO, which indeed would be only replacement therapy, if thiamine administration to thiamine-deficient organisms does in fact produce an MAO-inhibitor.

It is interesting that thiamine-deficient rats also show increased cholinesterase activity[15] and

diamine oxidase activity,[16] while thiamine is also a weak inhibitor of these enzymes, *in vitro*. It has been proposed that *in vivo* thiamine inhibits both of these enzymes.[15, 16] Thus, there is both precedent for the theory presented and a wider scope for a mechanism which would delay the catabolism of neurohumors such as acetylcholine, histamine, serotonin, and the catecholamines.

Department of Pharmacology,
Yale University School of Medicine,
New Haven, Conn., U.S.A.

H. Y. MELTZER

REFERENCES

1. H. Y. MELTZER, Unpublished observations.
2. I. B. WILSON and S. GINZBURG, *Biochim. Biophys. Acta.* **27**, 196 (1958).
3. E. M. GAL and P. A. DREWES *Proc. Soc. Exp. Biol., N.Y.* **106**, 295 (1961).
4. J. R. DI PALMA and P. HITCHCOCK, *Anesthesiology* **19**, 762 (1958).
5. S. K. WOLFSON JR. and S. ELLIS, *Fed. Proc.* **13**, 418 (1954).
6. S. B. GERTNER, *J. Pharmacol.* **131**, 223 (1960).
7. T. CESARMAN, *Ann. N. Y. Acad. Sci.*, **80**, 988 (1959).
8. H. WEISSBACH, T. E. SMITH, J. W. DALY, B. WITKOP, and S. UDENFRIEND, *J. Biol. Chem.*, **235**, 1160 (1960).
9. H. G. K. WESTERBRINK and J. GOUDSMIT, *Enzymologia* **10**, 146 (1941/42).
10. H. G. K. WESTERBRINK, *Proc. Fourth Int. Congr. Biochem.*, Vienna, Vol. XI, p. 72 (1959).
11. A. FUJITA, *Advanc. Enzymol.* **15**, 389 (1954).
12. A. VON MURALT, *Exp. Cell Res.*, Supp. 5, 72 (1958).
13. J. D. SPILLANE, *Biochemical Aspects of Neurological Disorders*, p. 17. Blackwell Scientific Publication, Oxford (1959).
14. C. A. ELVEHJEM, *J. Amer. Med. Ass.* **120**, 1388 (1942).
15. D. GLICK and W. ANTOPEL, *J. Pharmacol.* **65**, 389 (1939).
16. E. A. ZELLER, *Helv. Chem. Acta* **22**, 837 (1939).

Inhibition of dopamine β-oxidase by imipramine

(*Received* 11 *May* 1961)

PREVIOUSLY we have reported that phenylethylamines and phenylpropylamines inhibit the conversion *in vitro* of dopamine to norepinephrine by dopamine β oxidase. The conversion of dopamine to norepinephrine may be the rate-limiting step in the biosynthesis of norepinephrine and, consequently, inhibitors of dopamine hydroxylation may be important therapeutic agents in lowering norepinephrine levels or increasing dopamine levels *in vivo*. The present report shows that imipramine (N-(γ-dimethylaminopropyl)-iminodibenzyl), a known anti-depressant agent, inhibits the conversion *in vitro* of dopamine to norepinephrine.

The enzyme dopamine β-oxidase was prepared by the method of E. Y. Levine *et al.*,[1] and the incubation was carried out by the procedure described in the previous paper.[2] At the end of the period of incubation, the solution was analyzed for the enzymically formed norepinephrine by two different experimental procedures. In the first procedure, dopamine-C^{14} was used as a substrate, and after acetylation of the amines the enzymically formed norepinephrine-C^{14} was separated from dopamine-C^{14} by paper chromatography.[3] The amount of dopamine-C^{14} which disappeared from the incubation mixture, as well as the amount of norepinephrine-C^{14} formed in the incubation mixture, was calculated from the radioactivity of each of these compounds. In the second series, non-radioactive dopamine was used as a substrate and the enzymatically formed norepinephrine was determined by a modification of the fluoremetric method of von Euler and Floding.[4] The degree of inhibition rate of dopamine β oxidase was determined in both procedures by comparing the amount of norepinephrine formed in an incubation mixture which contained only the substrate and in an incubation mixture which contained imipramine and the substrate. Table 1 shows the effects of imipramine on the conversion of dopamine to norepinephrine. It is evident that when the concentration of inhibitor to substrate is 2:1 approximately 50 per cent inhibition of norepinephrine synthesis is observed.

Since inhibition of monoamine oxidase by imipramine was shown to be negligible[5] there is no basis for speculation along this line concerning its mode of action as a psychoenergizer. The present finding that imipramine inhibits dopamine β-oxidase may suggest that its pharmacological activity is a consequence of inhibition of norepinephrine synthesis which results in an increase of dopamine

TABLE 1. INHIBITION OF DOPAMINE TO NOREPINEPHRINE CONVERSION BY IMIPRAMINE*

Expt. no.†	Dopamine added (μ moles)	Imipramine added (μ moles)	Norepinephrine formed (μ moles)	Dopamine disappearing (μ moles)
1	0·4	none	0·12 ± 0·02	0·23 ± 0·05
1	0·4	2·00	0·07 ± 0·02	0·29 ± 0·05
2	4·00	none	1·46 ± 0·08	not estimated
2	4·00	4·00	1·43 ± 0·08	not estimated
2	4·00	8·00	1·00 ± 0·08	not estimated
2	4·00	12·00	0·75 ± 0·06	not estimated

* Figures represent averages of 3 experiments in each series.
† In Experiment 1, 0·02 ml of the standard enzyme solution was added to the incubation mixture and dopamine-C^{14} was used as a substrate. In Experiment 2, 0·2 ml of the standard enzyme solution was added and non-radioactive dopamine was used as a substrate. The final volume of each incubation mixture was adjusted with 0·02 N phosphate buffer, pH 6·4, to 1·3 ml.

levels in various organs. Some preliminary studies with animals treated with imipramine have shown that in such animals the dopamine levels in various organs were higher than in untreated control animals, while the norepinephrine levels in the time period thus far studied were not altered significantly. Whether imipramine is a substrate for dopamine oxidase, as has been shown for phenylethylamines and phenylpropylamines,[6] is under investigation.

Acknowledgements—This work was supported by grants from the National Institutes of Health, and Parkinson's Disease Foundation.

Department of Psychiatry and Neurology,
Neurochemistry Laboratory,
New York University College of Medicine,
New York, N.Y., U.S.A.

M. GOLDSTEIN
J. F. CONTRERA

REFERENCES

1. E. Y. LEVIN *et al.*, *J. Biol. Chem.*, **235**, 2080 (1960).
2. M. GOLDSTEIN and J. F. CONTRERA, *Biochem. Pharmacol.*, **7**, 77, (1961).
3. M. GOLDSTEIN *et al.*, *Proc. Soc. Exper. Biol. Med.*, **103**, 137 (1960).
4. U. S. VON EULER and I. FLODING, *Acta Physiol. Scand.*, **33**, 45 (1955).
5. P. GREENGARD, Private communication.
6. M. GOLDSTEIN *et al.*, *Experientia*, in press.

Biochemical Pharmacology, 1961, Vol. 7, pp. 280. Pergamon Press Ltd., Printed in Great Britain.

BOOK REVIEWS

P. J. HEALD: **Phosphorus Metabolism of Brain.** Pergamon Press Ltd., Oxford, 1960. pp. vii + 195, 19 fig. 42*s*.

THIS book brings an up-to-date survey of the literature on the metabolism of phosphorus-containing compounds in brain. There are two main groups of phosphorus compounds on which attention is focussed: the acid soluble labile energy-rich phosphates which supply the energy for many metabolic processes, and the phospholipids whose importance as tissue components is greater in the brain than in any other organ.

Study of the labile compounds requires special techniques such as freezing of whole animals in liquid air, and tracer techniques are needed for much of the work on lipid metabolism. From the biochemical point of view, *in vitro* work is more satisfactory than work on the whole organism, but meets with greater difficulties in the functional interpretation of the results.

The technique of using electrical pulses for the stimulation of brain slices is a first attempt at bridging the gap between the *in vitro* and *in vivo* world, and the study of the action of drugs on such systems has sometimes yielded results when the study of the unstimulated tissue slice did not. The recent work by Hokin and Hokin who found striking biochemical actions of acetylcholine and atropine *in vitro*, represents another attempt at narrowing that gap; this work, however, receives rather limited attention. The biochemist will be grateful to the author for compiling much literature and including an appendix on analytical methods.

M. VOGT

BOOKS RECEIVED

R. GOULDING: **Handbook of Dental Pharmacology and Therapeutics.** William Heinemann, London, 1960. 199 pp.

H. P. KUEMMERLE, A. SENN, P. RENTCHNICK and N. GOOSSENS: **Klinik und Therapie der Nebenwirkungen.** Geor Thieme Verlag, Stuttgart, 1960. 1286 pp.

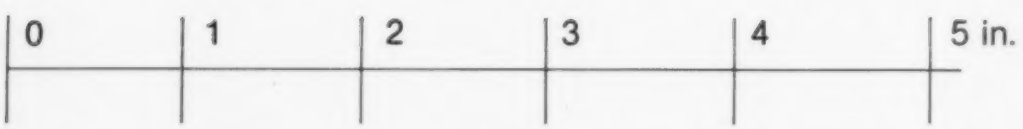
Vol. 7
1961

Vol. 8
1961

PERGAMON PRESS INC.

MICROFORMS INTERNATIONAL
MARKETING CORPORATION
FAIRVIEW PARK, ELMSFORD, NEW YORK 10523

l. 8
761



PRODUCED AT

**MIMC MICROFORMS LABORATORY
ELMSFORD, NEW YORK**